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Toxicity of Heavy Metals to Legumes and Bioremediation

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Preface

Rapid industrial operations and constantly dwindling fresh irrigation water sources have resulted in the increased use of industrial or municipal wastewater in agricultural practices, which quite often adds considerable amounts of heavy metals to soil. And therefore, metal concentrations sometimes present in soils commonly go beyond the threshold level, which after uptake by soil microbes including nodule bacteria, rhizobia, and plants such as legumes cause severe toxicity to both microbes and plants. In addition, heavy metals via food chain may cause human health problems also. Maintaining good soil quality is therefore of major practical importance for sustainable agronomic production. Contamination of agronomic soils with heavy metals and their consequent deleterious effects on the production systems have, therefore, received greater attention globally by the environmentalists.

   Among crops, legumes, which are grown largely in tropical and semiarid tropical regions, serve as a rich source of protein and provide a significant amount of nitrogen to soils. In addition, legumes are known to improve soil qualities, like organic matter, soil structure and porosity, fertility, microbial structure and composition, etc. In order to promote legume growth in varied ecosystems, microbes forming symbiosis with legumes and collectively called "rhizobia" are applied as inoculant to reduce dependence on chemical fertilizers frequently used in crop including legume production. Besides rhizobia, several other soil-inhabiting microbes possessing plant growth-promoting qualities, generally called as plant growth-promoting rhizobacteria (PGPR), have also been used and practiced as sole bioinoculant or as mixture with host-specific rhizobia for increasing the crop yields. These multipurpose organisms therefore broadly provide a practicable and low-cost substitute to compensate for alarmingly used synthetic chemical fertilizers in high-input agricultural practices in different production systems around the world for enhancing the quantity and seed quality of several crops including legumes. However, reports on the obvious toxicity of heavy metals to legumes and associated microflora and how such toxicities could be reduced/prevented employing inexpensive naturally abundant microbes are poorly documented. To circumvent the metal toxicity problems, several traditional physical and chemical methods have been applied, which, however, have not reached to optimum success level due to various socioeconomic or technical reasons. To overcome such barriers, there is therefore an urgent need to find an inexpensive and easily acceptable technology for metal cleanup from

contaminated sites. In this context, both rhizobia and legumes have been found to play important roles in restoring the metal-contaminated soils and subsequently in enhancing legume production in polluted environment. Considering on the one hand the importance of *Rhizobium*–legume interactions in maintaining soil fertility and metal toxicity to symbiotic relationships and the role of PGPR in metal detoxification on the other, grave efforts have been made to compile such demanding research in a single volume.

Toxicity of heavy metals to legumes and bioremediation presents numerous aspects of metal toxicity to legumes and suggests quite a few bioremediation strategies that could be useful in restoring contaminated environments vis-a-vis legume production in metal-stressed soils. The mobility and availability of toxic metals, nutritive value of some metals, and the strategies to assess the human health risk by heavy metals are reviewed and highlighted. Heavy metal toxicity to symbiotic nitrogen fixing microorganism and host legumes is dealt separately. A focused insight into the possible effects of heavy metals on seed germination and important physiological functions of plants including popularly grown legumes around the world have been amply reviewed and discussed in this book. The interaction between chromium and plant growth-promoting rhizobacteria and how chromium toxicity could be managed are explored. The influence of glutathione on the tolerance of *Rhizobium leguminosarum* to cadmium is covered in detail. The book further describes in a separate chapter, "Bioremediation: A natural method for the management of polluted environment," several bioremediation strategies commonly used in cleaning up the heavy metal-contaminated sites. "*Rhizobium*–legume symbiosis: A model system for the recovery of metal contaminated agricultural land" has been sufficiently discussed in this book. Microbially mediated transformations of heavy metals in rhizosphere are critically addressed. "Rhizoremediation: A pragmatic approach for remediation of heavy metal contaminated soil" is reviewed and highlighted. Plant growth-promoting rhizobacteria facilitate the growth and development of various plants in both conventional and stressed soils by one or combination of several mechanisms. This interesting aspect of PGPR in the management of cadmium-contaminated soil is dealt separately. The importance of mycorrhizal fungi in enhancing legume production in both conventional and derelict environment and site-specific optimization of arbuscular mycorrhizal fungi-mediated phytoremediation have been reviewed and discussed. Further in this book, heavy metal resistance in plants and putative role of endophytic bacteria are highlighted.

We indeed enjoy sharing especially with legume growers some of the most exciting developments in bioremediation and legume production in stressed environment and presenting this book as a key point of reference for everyone involved in research and development of legumes around the world. The data and methodologies described in this book are likely to underpin the development of sustainable legume production and serve as an important and rationalized source material. In addition, a broad perspective toward an issue of concern to researchers, students, professionals, policymakers, and practitioners in legume production in contaminated soil with minimum resources is highlighted. It would also serve as a valuable resource

for agronomists, environmentalists, soil microbiologists, soil scientists, biologists, and biotechnologists involved in the management of contaminated lands.

We are very grateful to our expert colleagues for providing their vital, reliable, and progressive information to construct this book. Chapters in this book are well explained with suitable tables and pictures, and contain most recent literature. We are undeniably very thankful to our family members for their constant and unrelenting support during the whole period of book preparation. And most of all, we are extremely thankful to our lovely children Zainab and Butool for helping us to avoid some tense moment during book preparation by their joyful activities. We are also very pleased with the book publishing team at Springer-Verlag, Austria, who always provided us their unconditional support in replying to all our queries very quickly. Finally, there may be a few basic errors/inaccuracies or printing mistakes in this book, for which we feel sorry in anticipation. However, if such mistakes are brought to our notice at any stage, we will certainly try to correct and improve them in subsequent print/edition. Any suggestion or decisive analysis of the contents presented in this book by the readers is welcome.

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Soil Contamination, Nutritive Value, and Human Health Risk Assessment of Heavy Metals: An Overview

1

Mohammad Oves, Mohammad Saghir Khan, Almas Zaidi, and Ees Ahmad

Abstract

Globally, rapidly increasing industrialization and urbanization have resulted in the accumulation of higher concentrations of heavy metals in soils. The highly contaminated soil has therefore become unsuitable for cultivation probably because of the deleterious metal effects on the fertility of soils among various other soil characteristics. In addition, the uptake of heavy metals by agronomic crops and later on consumption of contaminated agri-foods have caused a serious threat to vulnerable human health. Considering these, a genuine attempt is made to address various aspects of metal contamination of soils. In addition, the nutritive value of some metals for bacteria and plants is briefly discussed. Here, we have also tried to understand how heavy metals risk to human health could be identified. These pertinent and highly demanding discussions are likely help to strategize the management options by policy makers/public for metal toxicity caused to various agro-ecosystems and for human health program.

1.1 Introduction

The rapid industrial operations and consistently declining fresh irrigation water sources have led to the increase in use of industrial or municipal waste water in agricultural practices probably due to its (1) easy availability, (2) scarcity of fresh water, and (3) disposal problems. Even though sewage when applied provides water and valuable plant nutrients, it contributes sufficient amounts of heavy metals (HMs) to agricultural soils (Chen et al. 2005; Maldonado 2008; Zhang et al. 2008). In addition, heavy metals have been used over the years as building materials, in

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pigments for glazing ceramics, and pipes for transporting water, in batteries and other electronic products, and in painting (Horowitz 2009; Callender and Rice 2000). After discharge without proper care from various industrial sources or fertilizer application, HM accumulates in soils. Metal concentrations found in contaminated soils frequently exceed those required as nutrients or background levels, resulting in uptake by plants and deposition to unacceptable levels. When the level of HM goes beyond the permissible limits, they affect adversely the growth of beneficial soil microflora including nodule bacteria, rhizobia (Tyler 1993; McGrath et al. 1995; Paudyal et al. 2007). Furthermore, through food chain, HM cause problems to living organisms including microbes, plants, and humans/animals (Akoumianakis et al. 2009; Fu et al. 2009; Salvatore 2009; Zhang et al. 2008), as presented in Fig. 1.1. However, some of these metals which even may be there in foods such as iron and copper are essential as they affect many important biological systems. These elements can on the other hand be toxic for living organisms if their concentration is excessively high in the body. Other elements like mercury, arsenic, lead, and cadmium are not important; rather, they are toxic, even at fairly low concentration (Celik and Oehlenschlager 2007; Zarei et al. 2010). Despite these conflicting properties, metals in general have a unique ability to move and accumulate in various systems including precious but variable food chains over a period of time. The consistent and unchecked accumulations of
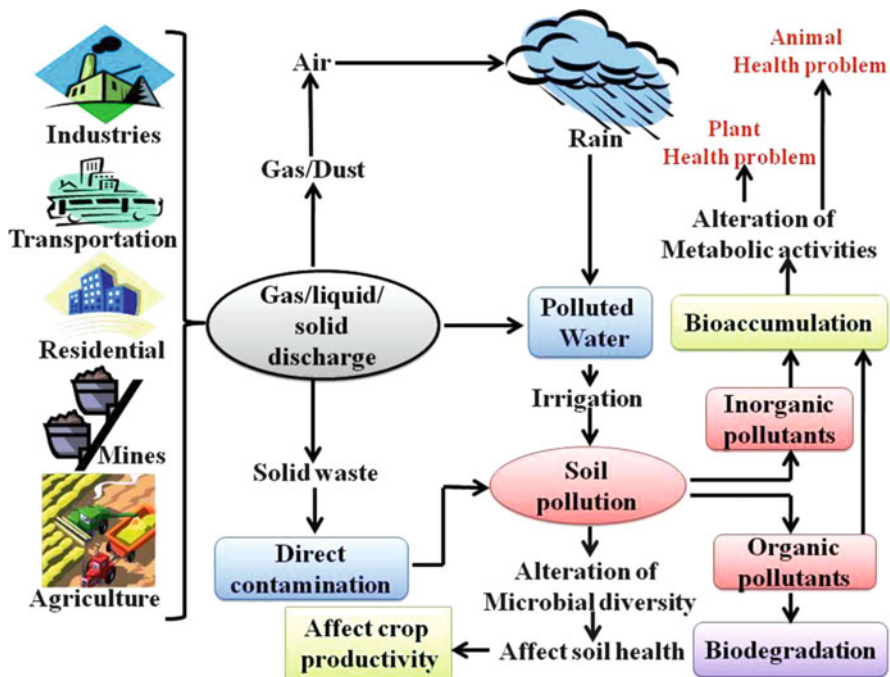
Fig. 1.1 Heavy metal contamination and its toxic effects on microbes, plants and animals

metals in the food chain damage different ecological niches and therefore pose a major threat to human health (Mishra et al. 2007; Efendioglu et al. 2007). For example, the consequence of certain metals has been reflected in the form of cancer, nervous system damage, and other diseases in humans (Zwieg et al. 1999).

1.2 Source of Heavy Metal in Soils

Heavy metal generally refers to metals and metalloids having densities greater than 5 g cm^{-3}. Heavy metals in soils may be found naturally or results from anthropogenic activities (Fig. 1.2). Natural sources include the atmospheric emissions from volcanoes, the transport of continental dusts, and the weathering of metal-enriched rocks (Ernst 1998). The other major source of contamination is anthropogenic origin: the exploitation of mines and smelters; the application of metal-based pesticides and metal-enriched sewage sludges in agriculture; the combustion of fossil fuel, metallurgical industries, and electronics (manufacture, use, and disposal); the military training, etc. (Alloway 1995).

According to Ross (1994), the anthropogenic sources of metal contamination can be divided into five major groups: (1) metalliferous mining and smelting (e.g., arsenic, cadmium, lead, mercury), (2) industry (e.g., arsenic, cadmium, chromium, cobalt, copper, mercury, nickel, zinc), (3) atmospheric deposition
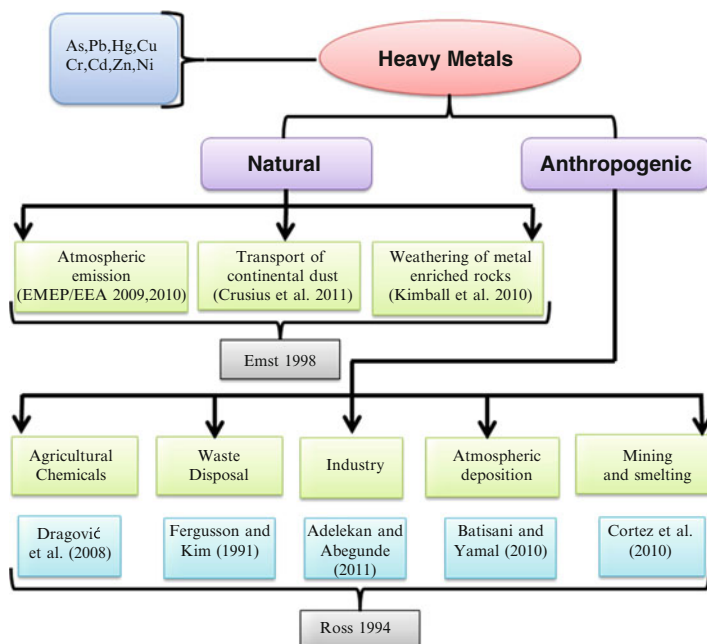
Fig. 1.2 Origin of soil contamination by heavy metals

(arsenic, cadmium, chromium, copper, lead, mercury, uranium), (4) agriculture (e.g., arsenic, cadmium, copper, lead, selenium, uranium, zinc), and (5) waste disposal (e.g., arsenic, cadmium, chromium, copper, lead, mercury, zinc). The use of intensive farm management practices, like application of phosphatic fertilizers (Mortvedt 1996; Nicholson et al. 2003), sewage sludge input, and pesticide treatments, are also the cause of soil pollution. Considering all source of origin, it is estimated that the annual worldwide release of heavy metals is about 22,000 tons (metric ton) for cadmium, 939,000 tons for copper, 783,000 tons for lead, and 1,350,000 tons for zinc (Singh et al. 2003). In 2009 alone, the total annual ferrochromium and chromite world production was 7,000,000 tons and 19,300,000 tons, respectively (USGS 2009).

Other source of soil pollution includes the emission of metals from heavy traffic on roads which may contain lead, cadmium, zinc, and nickel and are found in fuel as antiknock agents (Suzuki et al. 2008; Atayese et al. 2009). The deposition of vehicle-derived metal and the relocation of metals deposited on road surface by air and runoff water have led to contamination of soils (Nabuloa et al. 2006; Ogbonna and Okezie 2011). Road dust originating possibly from the emissions of electric arc furnace dust (EAFD) is reported to contain high concentrations of metals like Fe, Zn, Pb, and Cr (Sammut et al. 2006; Fernández-Olmo et al. 2007; Geagea et al. 2007). The serious wear and tear of tires and brake linings may also produce high concentrations of Fe, Zn, Cu, Cr, and Ni (Li et al. 2001; Adachi and Tainosho 2004; Iijima et al. 2007). The fly ash of coal-fired power plants contains metals like Fe, Ni, Cr, Cu, Zn, and Pb (Reddy et al. 2005; Gómez et al. 2007). Cadmium may be added to soil from sources like Cd-made compounds when used as stabilizers in PVC products, color pigment, several alloys, and also in rechargeable nickel–cadmium batteries. Industrial wastewater is yet other major metal contamination source of soils (Bergbäck et al. 2001; Sörme and Lagerkvist 2002). Contamination of soil may also result from dispersal and discharge of metals from various other sources. Such dispersal includes gas–dust release into the atmosphere from different technological processes requiring high temperature like power plants; metal smelting; burning of raw materials for cement, etc.; waste incineration; and fuel combustion. Another route of metal entry into soil is motor transport, which is widely connected with the use of lead as an additive to gasoline. Heavy metals in pristine river catchments originate from natural sources and processes as chemical weathering, soil erosion, fallout of natural aerosols from marine, and volcanic or arid soils sources (Avila et al. 1998; Gaillardet et al. 2003).

Contamination of agronomic soils with heavy metals and their adverse impact on the agro-ecosystems are therefore currently the focus of attention by the environmentalists around the world. This is because soil is an active and dynamic system where many chemical, physical, and biological activities are going on constantly. The massive interaction among living and nonliving components of soil determines the nutrient pool (fertility) of soil. Maintenance of good soil quality is therefore of prime importance for sustainable agriculture. However, the nutrient status of soil changes with time, prevailing conditions of climate and plant cover, and microbial composition of soil (Ademorati 1996). In addition, when some

stressors such as HM, temperature, extreme pH, or chemical pollution are imposed on a natural environment, soil biota can be affected, and whole ecological processes mediated by them are disturbed (MacGrath 1994; McGrath et al. 2005). Moreover, every 1,000 kg of "normal soil" contains 200 g chromium, 80 g nickel, 16 g lead, 0.5 g mercury, and 0.2 g cadmium, theoretically (IOCC 1996). Assessment of metal status in soils corresponding to pollution level is therefore of great practical interest due to their variable impact on different forms of water (groundwater and surface water) (Clemente et al. 2008; Boukhalfa 2007), microbial communities (Wani and Khan 2011), plant genotypes (Pandey and Pandey 2008; Stobrawa and Lorenc-Plucińska 2008), and animals and humans (Lagisz and Laskowski 2008; Korashy and El-Kadi 2008).

1.3   Metal Bioavailability

The total contents of metal present in soil do not provide any information regarding the availability and mobility of metals, while the assessment of metal availability is more important because it helps to better understand the specific bioavailability, reactivity, mobility, and uptake by plants (McBride 1994; Luo and Christie 1998). Based on the data available, metals present within soil have been categorized into five major geochemical forms as (1) exchangeable, (2) bound to carbonate phase, (3) bound to iron and manganese oxides, (4) bound to organic matter, and (5) residual metal. Metals found in any of these forms vary greatly in mobility, biological availability, and chemical behavior in soil probably because they react differently to various organic compounds such as low-molecular organic acids, carbohydrates, and enzymes secreted by microorganisms inhabiting soil (Huang et al. 2002). Also, the soil bacteria have charged surfaces which interact very strongly with metal ions in soil solution. They could absorb a greater amount of heavy metals than inorganic soil components such as montmorillonite, kaolinite, or vermiculite (Ledin et al. 1996). Bacterial cells have an extremely high capacity of adsorbing and immobilizing toxic ions from soil solution (Beveridge et al. 1995). In this context, Huang et al. (2000), for example, reported that symbiotic bacteria such as rhizobia when used as inoculant significantly increased the adsorption of Cu and Cd in soil. The mechanisms and adsorption kinetics are still poorly understood, regarding how bacteria affect the speciation and distribution of heavy metals in soils, especially under field conditions. Numerous methods like sequential extraction, single extraction, and soil column leaching experiments have been used to determine the possible chemical associations of metals in soils and to assess mobility and bioavailability of metals (Li and Thornton 2001; Cukrowska et al. 2004). Of the various methods employed, single extraction method which involves the use of a selective chemical extractant such as a chelating agent or a mild neutral salt (Ure 1996) is frequently used to indicate the bioavailability or mobility of heavy metals in a short or moderate term. The consequential extraction could provide valuable information for predicting metal availability to plants, metal movement in the soil profile, and transformation between different forms in soils in a long term (McGrath and Cegarra 1992). Batch or column leaching

experiment has also been used (Anderson et al. 2000) as a tool to assess the metal mobility in soil, sediment, and slag. This method can be applied to assess metal mobility and bioavailability that closely simulates the practical conditions (Cukrowska et al. 2004). Generally, the factors that affect the bioavailability and accumulation of heavy metals in soil/plants include (a) soil type, which includes soil pH, organic matter content, clay mineral, and other soil chemical and biochemical properties; (b) crop species or cultivars; (c) soil–plant–microbes interaction, which plays an important role in regulating heavy metal movement from soil to the edible parts of crops; and (d) agronomic practices such as fertilizer application, water managements, and crop rotation system. These factors together influence the thresholds for assessing dietary toxicity of heavy metals in the food chain, as reviewed by Islam et al. (2007).

1.4   Heavy Metal as Nutrient: An Overview

With ever increasing human populations, there is a continuous pressure on agricultural systems to produce more and more foods to fulfill the human food demands. To address these problems, well-directed and concerted efforts are required to efficiently use the full potential of agro-ecosystems. However, in agricultural practices, both major like nitrogen (N), phosphorus (P), and potassium (K) and minor nutrients play important roles in crop improvement. Apart from the major nutrients, the deficiency of micronutrients (which are typically present at <100 mg kg^{-1} dry weight) also limits the crop production severely in many production systems (Aghili et al. 2009). Some of the micronutrients essentially required for various metabolic activities of plants including legumes are copper, iron, manganese, and zinc. Even though these elements are required in smaller quantities by majority of plants, agricultural soils are usually deficient in one or more of these micronutrients. And hence, the concentration of these nutrient elements in plant tissues falls generally below the optimum levels. The minor elements, also called trace elements or other metalloids, play important roles in the functioning of living organisms and could participate in (1) forming structure of proteins and pigment, (2) redox processes, (3) regulation of the osmotic pressure, (4) maintaining the ionic balance, and (5) acting as enzyme component of the cells (Kosolapov et al. 2004). Among these elements, aluminum, cobalt, selenium, and silicon, for example, are known to promote plant growth and may be essential for particular taxa (Pilon-Smits et al. 2009). Also, some of these beneficial elements have been reported to enhance resistance to biotic stresses such as pathogens and herbivory and to abiotic stresses such as drought, salinity, and nutrient toxicity (Pilon-Smits et al. 2009). Similarly, zinc plays a vital role in the division and expansion of cells, protein synthesis, and also in carbohydrate, nucleic acid, and lipid metabolism (Collins 1981). On the other hand, when the concentrations of such trace elements rise above the normal threshold level, zinc, for example, inhibits the growth of both microbial communities (Wani and Khan 2011) and plants, for example, pea (Stoyanova and Doncheva 2002) and peanuts (Davis and Parker 1993; Davis et al. 1995).

The uptake of such elements differs from organisms to organisms (Beal et al. 2009) which, however, could be enhanced by increasing microbial biomass (Odokuma and Akponah 2010). The concentration of these trace elements also varies from soil to soil or region to region. For instance, the surveys conducted to determine the nutrient status of agricultural soils in China and India have revealed that zinc is the most common deficient micronutrient in soil. The levels of nutrient deficiencies in Chinese soils were (%) Zn 51, Mo 47, B 35, Mn 21, Cu 7, and Fe 5 (Zou et al. 2008), while in Indian soils, it were Zn 49, 33 B, 12 Fe, 11 Mo, 5 Mn, and 3 Cu (Singh 2008). Therefore, the understanding of the nutrient pool of soils and consequential impact of these elements both on microbes and plants are critical for improving the crop production and plant nutritional value for alarmingly increasing world populations.

1.4.1 Heavy Metals Importance in Microorganisms

Metals discharged from various sources followed by their deposition into soils and uptake by microbial communities affect directly and/or indirectly various stages of microbial growth, metabolism, and differentiation. The interaction of metals and their compounds with microbes, however, depends on the type of metal species, interacting organisms and their habitat, structural and biochemical compositions, and functional ability of the microbes (Khan et al. 2009a). These factors together influence the solubility, mobility, bioavailability, and toxicity of variously distributed metals in different locations (Gadd 2005, 2007). Some of the metals like copper, zinc, cobalt, and iron are essential for the sustenance but can exhibit toxicity when present above certain threshold concentrations probably because they form a complex with protein molecule which renders them inactive, for example, enzyme inactivation. On the other hand, some metals such as aluminum, cadmium, mercury, and lead, even though have no known important biological functions, could accumulate within cells and lead to variation in enzyme specificity, disrupt cellular functions, damage the DNA structure, and finally may result in cell death.

Nickel among metals, for example, is an essential nutrient and plays important roles in various cellular processes of microbes. Many microbes have the ability to locate nickel and absorb this element employing permeases or ATP-binding cassette-type transport systems. Once inside the cell, nickel is incorporated into several microbial enzymes like acetyl CoA decarbonylase/synthase, urease, aci-reductone dioxygenase, methylenediurease, NiFe hydrogenase, carbon monoxide dehydrogenase, methyl coenzyme M reductase, certain superoxide dismutases, and some glyoxylases (Hausinger 2003). At higher concentrations, nickel is, however, toxic to bacteria. To cope with such situation, bacteria have evolved certain strategies to regulate the levels of intracellular nickel as observed in two Gram-negative bacteria: *Escherichia coli* and *Helicobacter pylori* (Eitinger and Mandrand-Berthelot 2000; Mulrooney and Hausinger 2003). *Bradyrhizobium japonicum* HypB purified from an

overproducing strain of *Escherichia coli* has been shown to bind up to 18 nickel ions per dimer and also to contain GTPase activity (Fu et al. 1995). Another metal such as copper (a modern bioelement) exists in Cu^{2+} and Cu^+ forms and is considered one of the most important cofactor for various enzymes of higher organisms (Karlin 1993). In bacteria, washed cell suspensions of *Thiobacillus ferrooxidans* reduced Cu(II) to Cu(I) in the presence of S as a potential electron donor (Sugio et al. 1990); Cu(II) could be reduced under both aerobic and anaerobic conditions. However, only net reduction occurs under aerobic conditions when azide or cyanide is added to prevent the iron oxidase from oxidizing Cu(I). Copper reduction by *T. ferrooxidans* may play a role in copper leaching (Sugio et al. 1990). Similarly, under iron-deficient environment, plant growth-promoting rhizobacteria in general produce siderophores, a ferric iron-specific ligand, which are reported to increase plant growth by accelerating the access of iron within rhizospheric environment. For example, strains of *Rhizobium ciceri* able to form symbiosis specifically with chickpea (*Cicer arietinum* L.) produced phenolate-type siderophores such as salicylic acid and 2,3-dihydroxybenzoic acid. Although these compounds are produced in response to iron deficiency, nutritive components of the culture medium significantly affected their production. It seems that Cu(II), Mo (VI), and Mn(II) ions bound competitively with iron to siderophores, resulting in a 34–100% increase in production (Berraho et al. 1997).

There are certain metals which are also required during *Rhizobium*–legume symbiotic process. For example, cobalt is one such biologically essential microelement with a broad range of physiological and biochemical functions (Williams 2001; Balogh et al. 2003). Nevertheless, it becomes deleterious for many organisms when present at higher rates (Nies 1999). However, cobalt has been found associated with variable enzymatic activities in many organisms (Antonyuk et al. 2001; Kamnev et al. 2004) and can be located in magnetosomes (Vainshtein et al. 2002). Cobalt occurs mainly in the cofactor B12. Moreover, nitrile hydratase, a new class of cobalt-containing enzymes, has also been identified by Kobayashi and Shimizu (1998). For symbiotic association, cobalt is required for N_2 fixation in legumes and in root nodules of nonlegumes. Interestingly, the demand for cobalt is extremely greater for N_2 fixation than for ammonium nutrition. And if there is any deficiency, cobalt results in N deficiency symptoms. Therefore, whenever cobalt is applied, it has been observed to increase the formation of leghemoglobin, an essential component of N_2 fixation, and hence, it enhances the nodule numbers per plant and ultimately pod yield of legumes, for example, groundnut (Yadav and Khanna 1988). Among the various cobalamine-dependent enzyme systems of rhizobia involved in nodulation and N_2 fixation are methionine synthase, ribonucleotide reductase, and methylmalonyl coenzyme A mutase (Das 2000). The mixture of *Rhizobium* and cobalt has therefore been reported to significantly affect the total uptake of N, P, K, and Co by groundnut, when analyzed at harvest (Basu et al. 2006). Similarly, molybdenum forms the catalytic center of numerous enzymes which on the basis of cofactor composition and catalytic function have been grouped into two categories: (1) bacterial nitrogenases containing an FeMo-co in the active site and (2) pterin-based molybdenum enzymes. The second category enzyme includes sulfite oxidase, xanthine oxidase, and dimethyl sulfoxide reductase (DMSOR), each of which has distinct activities. Nitrate reductases, for

example, have been reported in *Desulfovibrio desulfuricans* (Moura et al. 2007) while aldehyde dehydrogenase in *D. gigas* (Moura and Barata 1994; Rebelo et al. 2000; Moura et al. 2004).

1.4.2 Some Examples of Metals Important for Plant Health

Generally, plant remains healthy as long as there is continuous supply of nutrients to them. However, whenever there is shortage of a nutrient, it results in symptoms of deficiency and, at very low supply, in early mortality. In contrast, the excess of any nutrient may cause injury and, at high levels, even death of plants. Plants require on the one hand the excess amounts of certain elements called as macronutrients: C, H, N, O_2, P, S, etc.; in addition, they also require chemical elements which are necessary in small amounts and are called micronutrients. These include B and Cl, and the metals Cu, Fe, Mn, Mo, Ni, and Zn. The nutrients belonging to both categories are found in varied agro-ecological niche. A few plants living in symbiosis with nitrogen-fixing microorganisms also require Co as nutrient. However, so far, metal as nutrient is concerned; there are two criteria which are used to define a metal as essential for plant health: (1) it is required by the plants to complete its life cycle, and (2) it is part of a molecule of an essential plant constituent or metabolite. Since the plants are autotrophs and use light energy during photosynthesis to convert H_2O and CO_2 into energy-rich carbohydrates and O_2, the growth and development of plants in general depend exclusively on photosynthesis, which, in turn, is dependent on a sufficient supply of numerous chemical elements, including metals like Cu, Fe, and Mn. Heavy metals and metalloids can enter plants via uptake systems including different metal transporters (Eide 2004; Perfus-Barbeoch et al. 2002). However, if there is any deficiency of metal, plants increase the metal availability in the root environment by lowering the pH through root exudates which may contain organic acids, or through release of metal-complexing agents. After the proper and sufficient supply is maintained, a signal from the shoot to the root stops the exudation process. Once they enter the plant systems, some metals when present at lower rates have been found to affect plant growth by participating in redox reaction and sometimes directly becoming an integral part of enzymes (Baker and Walker 1989). For example, zinc is required to maintain the integrity of ribosome, is needed in the formation of carbohydrates, catalyzes the oxidation processes in plants, and plays important role in the synthesis of macromolecules (Alloway 2009; Pandey et al. 2006). Similarly, manganese plays an important role in reactions of enzymes like malic dehydrogenase and oxalosuccinic decarboxylase. It is also needed for water splitting at photosystem II and for superoxide disumutase. In plants, cobalt complex is found in the form of vitamin B_{12} while iron is an essential element in many metabolic processes and is indispensable for all organisms.

1.5   Heavy Metal Toxicity: A Brief Account

1.5.1   Effects of Heavy Metals on Microbial Diversity

Changes in microbial community structure in response to metals are considered an important indicator of the biological availability and activity of metals within soil ecosystem. In this regard, heavy metals such as Cd, Pb, and Cd/Pb mix using the $CdSO_4$ and $Pb(NO_3)_2$ solutions at different application rates have been found to exhibit toxicological effects on soil microbes which led to the decrease in their numbers, and enzyme activities like acid phosphatase (ACP) and urease (URE). Frostegard et al. (1993) also reported a gradual change in microbial community structure which was based on variation in phospholipids' fatty acid profiles, when organisms were analyzed from metal-contaminated soils. However, the response of microbial communities to various metals varies with solubility and consequently the bioavailability and toxicity of metals in soil which in effect are influenced greatly by sorption, precipitation, and complexation ability of soils (van Beelen and Doelman 1997; Oste et al. 2001). Moreover, the interaction of metals with soil depends strongly upon physicochemical properties of soil, which may differ among various agro-climatic regions of the world. One of the first observations of metal toxicity to soil microorganisms in the Woburn Market Garden experiment was a strong decrease in the amount of soil microbial biomass (Brookes and McGrath 1984). Later on, this type of study was confirmed by Barajas-Aceves (2005) who suggested that the decrease in the total amount of biomass was due to decrease in the substrate utilization efficiency of microbes when subjected to metal stress (Chander and Joergensen 2001; Chander et al. 2002). The reduction in microbial biomass is considered as an indicator of metal pollution, but its suitability in environmental monitoring as an indicator of soil pollution is restricted because of its high spatial variability (Broos et al. 2007) and shortcomings in its measurement (Dalal and Henry 1986). Decline in the amount of microbial biomass has also been found associated with changes in community structure (Abaye et al. 2005; Khan et al. 2010) and often to increased metal tolerance, even with small amounts of metal contamination (Witter et al. 2000). The resulting effects of metal toxicity on different microbial communities inhabiting varied agro-ecosystems may be due to changes in the metal-sensitive ability of populations or community. However, no distinct threshold for metal toxicity is reported, but such thresholds may be site specific as observed by Bunemann et al. (2006).

1.5.2   Heavy Metals–Plants Interactions

Heavy metals at higher concentrations cause severe damage to the various metabolic activities leading consequently to the death of plants including those of legumes, for example, green gram (Fig. 1.3A), pea (Fig. 1.3B), and chickpea (Fig. 1.3C). However, some plant species possess the ability to survive in soils even contaminated heavily with metals (Kneer and Zenk 1992). Metal at exceedingly
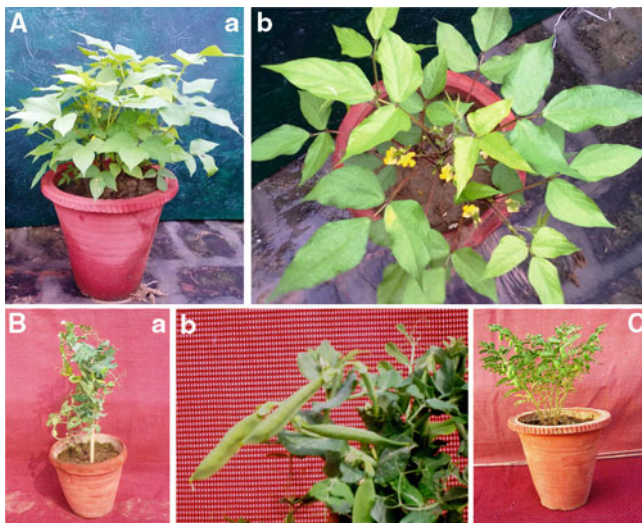
Fig. 1.3 Growth of greengram (**A** (a) vegetative growth and (b) with flowering), pea (**B** (a) vegetative growth and (b) with pods), and chickpea with seed pods (**c**) grown in conventional soils

higher concentrations is reported to damage plants by (1) inhibiting physiologically active enzymes (Stobart et al. 1985), (2) inactivating photosystems (Clijsters and Van Assche 1985; Somasundaram et al. 1994; Pandey and Tripathi 2011), and (3) disturbing mineral metabolism (Gadd 2007, 2010). In yet other study, Sandmann and Bflger (1980) have pointed out the importance of lipid peroxidation by metal (e.g., Cu) stress. Under nutrient deficient soil, the solubility of organic carbon and concomitantly the mobility of contaminants or pollutants such as heavy metals are increased. Dissolved soil organic matter has the significant effects on transformation of heavy metals through the increment of heavy metal solubility, root growth, and plant uptake (Quartacci et al. 2009; Kim et al. 2010). Copper and Pb accumulation in maize (*Zea mays* L.) and soybean (*Glycine max* L.) as affected by application of plant nutrients in soil such as N, P, and K (Xie et al. 2011) resulted in reduction in photosynthesis, stomatal conductance, and biomass while cadmium application caused a decline in the net rate of photosynthesis, stomatal conductance, and biomass in pak choi and mustard (Chen et al. 2011) but increased total chlorophyll content in tomato and decreased total biomass (Rehman et al. 2011). Accumulation of Zn and Cd in roots, petioles, and leaves of *Potentilla griffithii* was increased significantly with addition of these metals individually while Zn supplement decreased root Cd accumulation but increased the concentration of Cd in petioles and leaves (Qiu et al. 2010). The protective effect of Mg against Cd toxicity could in part be due to the maintenance of Fe status or to the increase in antioxidative capacity, detoxification, and/or protection of the photosynthetic apparatus (Hermans et al. 2011).

1.5.3 Metal Impact on Human Health

Heavy metals after release from various sources may enter into soil, vegetation, and water depending on their density. After their deposition in various systems, metals cannot be degraded and therefore persist in the environment causing human health problems through inhalation, ingestion, and skin absorption. On the other hand, heavy metals have willingly been used by humans for quite long times in metal alloys and pigments for paints, cement, paper, rubber, and other materials and are increasing even today in some parts of the world despite their well-known adverse effects. Acute exposure to metals may lead to nausea, anorexia, vomiting, gastrointestinal abnormalities, and dermatitis. Heavy metal toxicity can also damage or decrease mental and central nervous function (Gybina and Prohaska 2008), and damage blood composition (Cope et al. 2009), lungs (Kampa and Castanas 2008), kidneys (Reglero et al. 2009), livers (Sadik 2008), and other important organs (Lindemann et al. 2008; Lovell 2009). Furthermore, the long-term exposure of heavy metals may slowly impair physical, muscular, and neurological degenerative processes similar to Alzheimer's disease (Kampa and Castanas 2008), Parkinson's disease (Crawford and Bhattacharya 1987), and muscular dystrophy and multiple sclerosis (Turabelidze et al. 2008). High exposure can also lead to obstructive lung disease and has been linked to lung cancer, and damage to human's respiratory systems. In contrast, some metals like copper, selenium, and zinc (trace elements) play an important role in maintaining the metabolism of the human body. Copper, for example, is an essential substance to human life, but in high doses, it can cause anemia, liver and kidney damage, and stomach and intestinal irritation.

1.6 Human Health Risk Assessment: A General Perspective

Contamination of soils by heavy metals followed by uptake of metals through various agencies like foods, feeds, water, etc. (Marshall et al. 2007; Sharma et al. 2007; Khan et al. 2008a, b; Sridhara Chary et al. 2008; Zhuang et al. 2009a, b), by humans has become one of the most serious environmental problems that has threatened the precious human health (Eriyamremu et al. 2005; Muchuweti et al. 2006; Moore et al. 2009). Therefore, there is indeed an urgent and collective effort required to clean up the contaminants from environment so that the risk of metal toxicities could completely or at least to some extent be minimized. The concern resulting from the potential exposure of populations vulnerable to toxicants has, however, forced workers of different disciplines to act together in order to develop methodologies so that the actual impact of heavy metals on both the varying environment and the human health could be assessed (Eriyamremu et al. 2005; Muchuweti et al. 2006).

1.6.1   What Is Human Health Risk Assessment?

A human health risk assessment is in fact the method of assessing the probability of harm caused to people resulting from exposure to contaminants at a site. And therefore, both the deleterious (toxic) effects of pollutants and the ways that people may be exposed to these substances are evaluated.

In this context, for evaluating the risk caused by heavy metals, different workers apply different approaches (Baes et al. 1984; Sauvé et al. 1998; Hough et al. 2003, 2004). However, the role of both scientists (risk assessors) and decision makers (risk managers) in the evaluation process is central to the understanding of the risk assessment. In general, two approaches can be applied for evaluating the risk of a specific pollutant to any individual population: direct approach (biological) and indirect (environmental monitoring). For example, different human biomonitors, like plasma and urine, human milk, hair, and adipose tissue, may be used in surveillance programs. Even though these sources may provide real and direct information about how population is exposed to pollution, they are variable and depend largely on personal characteristics, such as dietary habits, smoking, weight, etc., rather than on low-level environmental exposures (Paustenbach et al. 1997). On the other hand, the chemical analysis of the pollutant concentrations originating from different sources like air, soil, vegetation, sediment, etc., may be an interesting indirect methodology for human health risk assessment. However, in order to make chemical methods more viable and effective, it should be complemented with biological and toxicological methods (Vaajasaari et al. 2002; Tsui and Chu 2003; Robidoux et al. 2004; Gruiz 2005). Considering these, it is generally believed that health risk assessment may play an important role in protecting humans from the nuisance of heavy metals.

1.6.2   Why We Do Assessment and What Is Risk Assessment Process?

Risk assessment strategies often aimed at populations are a systematic and multi-step process which is used to determine the magnitude, likelihood, and uncertainty of environmentally induced health effects (Sexton et al. 1995). Risk assessment has thus been suggested as a process which is generally used to collect scientific information regarding the toxicants and providing it to the policy/decision makers so that the human exposures to these substances could be regulated and managed. Broadly, risk assessment process includes four steps:

(a) Hazard Identification. In this step, site data relevant to human health are gathered and analyzed. And if there is any effect, that effect is again monitored to see whether it requires any further scientific investigations or not. For this, various tools, like quantitative structure–activity relationship (QSAR), short-term toxicity test, etc., are used in order to estimate the chemical damage of a single substance. However, this process also depends upon the origin of hazardous substances in question. For example, when establishing the hazard from

industrial sources, the chemicals are also identified according to the measurements of amount and typology of emissions.

(b) Dose–Response Assessment. The quantitative relationships between the magnitude of the exposure (or dose) and the probability of occurrence of adverse effects of toxicants on the population are critically examined in this step of risk assessment. Generally, the doses higher than normal are used to determine the toxic efficiency of a particular substance.

(c) Exposure Assessment. During this stage, the extent of magnitude of actual and/or exposure of the population to the hazardous agent in question is quantitatively determined.

(d) Toxicity Assessment. It involves the evaluation of adverse health effects resulting from the exposure to different metals.

(e) Risk Characterization. After collecting data from the earlier components, the information obtained is used later on to determine the nature and magnitude of the risk. This information subsequently helps policy/decision makers and other public to adopt proper protection measure against a particular pollutant.

1.6.3 Why Food Materials Are Used for Human Health Risk Assessment?

This is a reality that food safety has always been a serious and significant public concern globally. On the other hand, providing contamination free/safe foods to the constantly increasing human populations has been a bigger challenge before the scientist due to declining land resources which could be due to heavy metal poisoning among various other reasons (D'Mello 2003; Gholizadeh et al. 2009). We are aware of the facts that heavy metals without doubt persist in the environment and cannot be destructed due to which their concentrations increase to toxic levels (Bohn et al. 1985). In addition, the biological half-lives of heavy metals are generally long, and they have the ability to move and concentrate in various body organs leading to undesirable problems (Sathawara et al. 2004; Ata et al. 2009). Contamination of plant edible parts, for example, seeds, whole plants, or other plant products, obtained from metal-contaminated soil followed by their uptake by humans and other animals basically constitute an important route of metal exposure (Jackson and Alloway 1992; Wang et al. 2005). These metals affect both the nutritive value of food materials, and when it enters inside the human bodies, the higher concentration of heavy metals causes problems to human health which even sometimes may result in death (Mushak et al. 1989; Reilly 1991; WHO 1992a, b; Waalkes and Rehm 1994; Steeland and Boffetta 2000). Due to these enigmas, efforts have been directed by the scientists to understand the gravity of difficulties and to develop methods to accurately pinpoint the potential risks to populations living on and consuming food materials grown in metal-contaminated environment.

1.6.3.1 Vegetables as a Model Food for Human Health Risk Assessment: Why?

Vegetables are one of the important components of human dietary system around the world. However, when grown in metal-contaminated soils, many vegetables including lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) have been reported to accumulate cadmium, copper, zinc, and manganese in various organs (Intawongse and Dean 2006). The concentrations of elemental cadmium, zinc, and nickel in vegetables like *Beassica pekinensis* (L.), *Allium fistulosum* (L.), and *Spinacia oleracea* (L.) collected from the wastewater-irrigated soils exceeded the maximum permissible limits, and this also increased the daily intake of metals (DIM) by food (Xue et al. 2011). Similarly, the edible shoots of vegetables like *Gynandropsis gynandra* had the highest concentration of cadmium, lead, and copper, while *Amaranthus dubius* contained the highest zinc concentration, when grown in farmers' gardens situated on nine contaminated sites, used to grow vegetables for commercial or subsistence consumption in and around Kampala City, Uganda (Nabulo et al. 2010). The major source of metals in such vegetables was the irrigation with wastewater, effluent discharge from industry, and dumping of solid waste in such cultivation area. Of these metals, cadmium concentration was consistently lowest in *Cucurbita maxima* and *Vigna unguiculata*, indicating that these vegetables were able to prevent the uptake of cadmium from contaminated soil. After consumption, their presence has been observed in human gastrointestinal tract from the edible part of vegetables using an in vitro gastrointestinal (GI) extraction technique (Intawongse and Dean 2006). However, the bioavailability of metals in vegetables depends largely on the nature of vegetables, and some of them have a greater potential to accumulate higher concentrations of heavy metals than others. When metal containing vegetables are consumed, it may lead to various chronic diseases such as emphysema, bronchiolitis and alveolitis, and renal effects or may lead to impairment of growth and reproduction; reduction in the hemoglobin synthesis; disturbance in the functioning of kidney, joints, and reproductive and cardiovascular systems; and chronic damage to the central and peripheral nervous systems (European Union 2002; Nolan 2003; Ogwuegbu and Muhanga 2005; Young 2005). Due to these, vegetables have been used as a model system to assess the risk of heavy metals to human populations across different regions (Khan et al. 2009b; Zhuang et al. 2009a, b).

1.6.4 What Are Different Risk Assessments Methods?

Different methods like hazard quotient (HQ), health risk index (HRI), DIM, and daily dietary index (DDI) for assessment of the heavy metal concentrations in the human body following consumption of contaminated vegetables are briefly discussed.

1.6.4.1 Hazard Quotient

This is a ratio of the average daily dose (ADD) to the reference dose (R_fD). According to this, if the HQ ratio is less than 1, there is no risk to population but if the ratio is equal or greater than 1, then the ADD of particular metal is greater than R_fD, indicating that population is likely to have health risk due to that metal and therefore requires toxicity management option. This risk assessment method has been used by many workers (Chary et al. 2008; Chien et al. 2002; Wang et al. 2005) and was found valid and accurate. For calculating the HQ, following equation is used:

$$\text{HQ} = \left[W_{\text{plant}}\right] \times \left[M_{\text{plant}}\right]/R_fD \times B,$$

where $[W_{\text{plant}}]$ is the dry weight of contaminated plant material consumed (mg kg^{-1}), $[M_{\text{plant}}]$ is the concentration of metal in vegetables (mg kg^{-1}), R_fD is the food reference dose for the metal (mg d^{-1}), and B is the body mass (kg). The values of R_fD for heavy metals are taken from Integrated Risk Information System (Gholizadeh et al. 2009) and Department of Environment, Food and Rural Affairs (DEFRA 1999). Applying HQ, risk of consuming metal-contaminated grain and vegetables to human health has been assessed by various workers (Zheng et al. 2007; Zhuang et al. 2009a, b; Yang et al. 2011). For example, the risk to human health, expressed as a "hazard quotient" (HQ$_M$), was generally greatest for cadmium, followed successively by lead, zinc, nickel, and copper accumulating leafy vegetables like *Gynandropsis gynandra*, *Amaranthus dubius*, *Cucurbita maxima*, and *Vigna unguiculata* grown in metal-contaminated farmers' gardens of Uganda (Nabulo et al. 2010). Nevertheless, it was apparent that urban cultivation of leafy vegetables could be safely pursued on most sites, subject to site-specific assessment of soil metal burden, judicious choice of vegetable types, and adoption of washing in clean water prior to cooking. Similarly, the health risk of metals such as lead, cadmium, nickel, and chromium via consumption of greenhouse cucumbers and bell peppers produced in Iran using the total noncancer hazard quotient (THQ) and cancer risk assessment estimates was studied (Aghili et al. 2009). The individual metal THQ values indicated that there was no cancer health effects associated with intake of a single metal via consumption of either cucumbers or bell peppers. The THQ for all population groups which consumed greenhouse cucumbers and bell peppers was less than 1. This value indicated a low possibility of any obvious risk. However, among metals, cadmium was identified as the major risk factor for the consumers. The cancer risk assessment for lead for Qom adult populations groups via consumption of cucumber and bell peppers was greater than 1×10^{-6}. Higher lead and cadmium levels in the greenhouse vegetables were found as a major concern that requires immediate attention. In other study, the THQ and HI were calculated to evaluate the noncarcinogenic health risk from individual heavy metal (e.g., Hg, Pb, Cd, Zn, and Cu) and combined heavy metals due to dietary intake by adults and children in the industrial area of Huludao City, northeast of China (Zheng et al. 2007). Target hazard quotients for single heavy metal following intake

of individual foodstuff (e.g., cereal, sea product, vegetable, fruit, milk, bean, and egg) in the industrial area of Huludao was <1, indicating that there was no health risk and use of only one kind of foodstuff (e.g., vegetable) was safe. The use of multiple foodstuff may, however, lead to potential health risks for children and adults since HIs for heavy metals following dietary intake were >1. The relative HIs for Hg, Pb, Cd, Zn, and Cu were 1.7%, 11.7%, 24%, 23.4%, and 39.6% for adults and 1.5%,11.7%, 21.8%, 26%, and 38.8% for children, respectively.

1.6.4.2 Daily Dietary Index

Since many foods or food products do contain heavy metals if collected from metal-contaminated environment, their daily intake by humans requires to be evaluated consistently for comparison as suggested by US-EPA. DDI is determined simply by applying the formula:

$$DDI = X \times Y \times Z/B,$$

where X = metal in vegetables, Y = dry weight of the vegetables, Z = approximate daily intake, and B = average body mass of the consumers.

1.6.4.3 Daily Intake of Metals

This is evaluated by the equation:

$$DIM = C_{metal} \times C_{factor} \times D_{food\ intake}/B_{average\ weight},$$

where C_{metal} = heavy metals concentrations in plants (mg kg^{-1}), C_{factor} = conversion factor, and $D_{food\ intake}$ = daily intake of vegetables.

The conversion factor of 0.085 is to convert fresh vegetable weight to dry weight (Rattan et al. 2005). Following this method, Yang et al. (2011) determined the concentration and daily intake (DI) of heavy metals like, Pb, Zn, Mn, Cu, Cd, and Cr, in market vegetables in Chongqing of China. Also, the potential health risk to local consumers was evaluated by calculating THQ. The observed values for Pb and Cd were greater than those of safety limit fixed by FAO/World Health Organization (WHO) and Chinese regulations, indicating that market vegetables were seriously contaminated by tested metals. The DI values for Pb, Mn, and Cd were also above the international guideline bases, and hence, consumers were at higher health risk. The individual THQ for Pb and Cd in pak choi and Cd in mustard and the combined THQ for all metals in each vegetable species excluding lettuce were above the threshold 1, implying the obviously adverse effect on health.

1.6.4.4 Health Risk Index

By using DIM values and reference oral dose, the HRI is calculated as HRI = DIM/RD_f; if the calculated HRI value is less than 1, the exposed population is considered safe (IRIS 2003). Following this method, the health risks of heavy metals like chromium, copper, and zinc in edible seeds of crops grown in sewage-irrigated soils located

in the Langfang of Hebei province, China, were assessed. The HRI values for each heavy metal except copper following intake of the edible seeds were less than 1, suggesting that the crops grown in sewage-irrigated soil did not pose any health risk to human and therefore were considered safe for human consumption (Chen et al. 2010). In a similar study, Khan et al. (2008a, b) evaluated the health risks of heavy metals in food crops grown in soil irrigated with wastewater. A substantial concentration of heavy metals was build up in wastewater-irrigated soils, collected from Beijing, China. Also, the heavy metal concentrations were significantly higher in plants grown in wastewater-irrigated soils compared to those observed for plants grown in untreated soil. Interestingly, the plants grown in wastewater-irrigated soil had heavy metals greater than the permissible limits set by the State Environmental Protection Administration (SEPA) in China and the WHO. In addition, both adult and child populations tested in this study had significant amount of the metals, when they were allowed to consume crops grown in wastewater-irrigated soil. The HRI values were, however, less than 1 which was suggestive of the fact that there were no health risks of these groups even when they consumed contaminated vegetables. In a follow-up study, the HRI values have also been found less than 1 for food crops such as *Brassica rapa*, *Spinacia oleracae* (L.), *Lycopersicum esculantum*, *Mentha viridis*, *Coriandum sativum,* and *Lactuca sativa*, grown in wastewater-irrigated soil containing zinc. However, such crops had a fair chance of posing health risk, when grown in wastewater-irrigated soil containing higher concentration of Mn (Jan et al. 2010). Risk to human health by heavy metals like cadmium, copper, lead, zinc, nickel, and chromium, after consuming vegetables and cereal crops collected from wastewater-irrigated sites, was assessed by Singh et al. (2010). When analyzed, it was observed that all the collected samples from wastewater-irrigated sites had significantly higher concentrations of metals compared to those grown with clean water only. Of the various metals determined, the levels of cadmium, lead, and nickel were above the "safe" limits of Indian and WHO/FAO standards in all the vegetables and cereals. Furthermore, the higher metal pollution index and HRI values suggested that human populations who may consume these food materials collected from wastewater-irrigated site are likely to experience health related problems.

Conclusion

Soil contamination by heavy metals leading through various stages to human health problems or problems to other agro-ecosystem has been one of the prime concerns of environmentalists. Considering the threat of heavy metals to soil fertility, food safety, and human health at large, efforts are being directed to reduce/completely alleviate metal toxicity to all forms of life. However, further efforts are required to carefully monitor and regulate the discharge of properly treated by-products of different industries or to avoid the use of substances that otherwise contains heavy metals, in agricultural practices. By doing this, the uptake of metals by various plants and, hence, by the food materials could be avoided. Moreover, methods should be developed to rapidly identify the presence of toxic substances in consumable food items so that a suitable strategy is

adopted in time to eliminate human health problems. Therefore, all these require concerted efforts from public, scientists, and policy makers to achieve a common goal of "safe and secure environment" for better living around the world.

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Heavy Metal Toxicity to Symbiotic Nitrogen-Fixing Microorganism and Host Legumes

Ees Ahmad, Almas Zaidi, Mohammad Saghir Khan, and Mohammad Oves

Abstract

Legume species of the flowering family *Fabaceae* are well known for their ability to fix atmospheric nitrogen and enhance nitrogen pool of soil, leading to increase in crop especially legumes both in conventional or derelict soils. The interaction between Rhizobia and legumes provides nutrients to plants, increases soil fertility, facilitates plant growth and restores deranged/damaged ecosystem. These characteristics together make legume extremely interesting crop for evaluating the effect of heavy metals. Environmental pollutants like heavy metals at lower concentrations are required for various metabolic activities of microbes including Rhizobia and legume crops. The excessive metal concentrations on the other hand cause undeniable damage to Rhizobia, legumes and their symbiosis. Currently, little is, however, known about how free-living Rhizobia or the legume–*Rhizobium* symbiosis is affected by varying metal concentration. We focus here that how the nitrogen-fixing root nodule bacteria, the "rhizobia," increase plant growth and highlight gaps in existing knowledge to understand the mechanistic basis of how different metals affect rhizobia–legume symbiosis which is likely to help to manage legume cultivation in metal contaminated locations.

2.1 Introduction

Heavy metals discharged from industrial operations and upon consequent accumulation in various ecological systems cause a massive threat to the varied agroecosystems (Ceribasi and Yetis 2001; Cheung and Gu 2007). When heavy metals accumulate into soil to an abnormal level, it causes dramatic changes in microbial composition and

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their activities (Paudyal et al. 2007; Wani et al. 2008a; Khan et al. 2009a; Krujatz et al. 2011), leading consequently to losses in soil fertility. As a result of depleted soil nutrient pools resulting from direct or indirect metal effect, the health of plants including legumes like greengram [*Vigna radiata* (L.) Wilczek] (Fig. 2.1a) (Wani et al. 2007a), pea (*Pisum sativum* L.) (Fig. 2.1b) (Wani et al. 2008a) and chickpea (*Cicer arietinum* L.) (Fig. 2.1c) (Wani et al. 2008c; Wani and Khan 2010) growing in metal-enriched soil is adversely affected either due to nutrient deficiency or due to direct effects of toxicants. For instance, the higher concentrations of metals have shown toxicity to various physiological processes like synthesis of chlorophyll pigments in various plants (Feng et al. 2010) including legumes (Bibi and Hussain 2005; Wani et al. 2007b, c; Ahmad et al. 2008a), inactivated protein synthesis (Van Assche and Clijsters 1990; Brahima et al. 2010) and consequently led to the severe reduction in crop yields (Wani et al. 2007a, 2008b). In addition, there are numerous reports where elevated amounts of heavy metals have been found to limit the rhizobial growth and their host legumes (Heckman et al. 1987; Broos et al. 2005) and concomitantly reduce the crop yields (Moftah 2000). For example, a single strain of *Rhizobium leguminosarum* could survive well in the metal contaminated plots, but this strain did not fix N with white clover (*Trifolium repens* L.), although it resulted in N formation with *Trifolium subterraneum* (Hirsch et al. 1993). In a similar manner, a profound toxic effect of metal on N_2 fixing ability of culture inoculated white clover was observed (Broos et al. 2004). In other reports, when sludge was applied for field trials in Braunschweig, it was found that the increasing sludge rates reduced the number of indigenous populations of *R. leguminosarum* bv. *trifolii* to low or undetectable levels (Chaudri et al. 1993). Similarly, adverse effect of sludge application on N_2 fixation in faba bean (*Vicia faba*) (Chaudri et al. 2000) is reported. The reduction in growth and symbiosis in white clover were due to cadmium, lead and zinc, when plants were grown in soils highly contaminated with these metals (Rother et al. 1983).

2.2 What Are Nitrogen-Fixing Microbes?

All organisms capable of transforming atmospheric dinitrogen to biologically available form of N, for example, ammonia through a process called biological nitrogen fixation (BNF), are in general collectively referred to as nitrogen-fixing organisms (NFO). Among the two most widely studied nitrogen-fixing groups, asymbiotic represented for example by *Azotobacter* spp. (Plate 2.1a) and symbiotic bacteria (Plate 2.1b) capable of forming nitrogen-fixing organ nodules (Fig. 2.2) on leguminous plants have classically been named "Rhizobia." In the beginning, all bacteria able to nodulate legumes were included in a single genus, *Rhizobium* (Frank 1889), within the family Rhizobiaceae (Conn 1938). This genus had four fast-growing species: *R. leguminosarum* (Frank 1889), *R. phaseoli*, *R. trifolii* and *R. meliloti* (Dangeard 1926) and two slow-growing species: *R. japonicum* (Buchanan 1926) and *R. lupini* (Eckhardt et al. 1931). Later, on the basis of infection data, *R. leguminosarum* was found as microsymbiont for *Vicia*, *Pisum* and *Lens*; *R. phaseoli* for *Phaseolus*; *R. trifolii* for *Trifolium*; and *R. meliloti* for
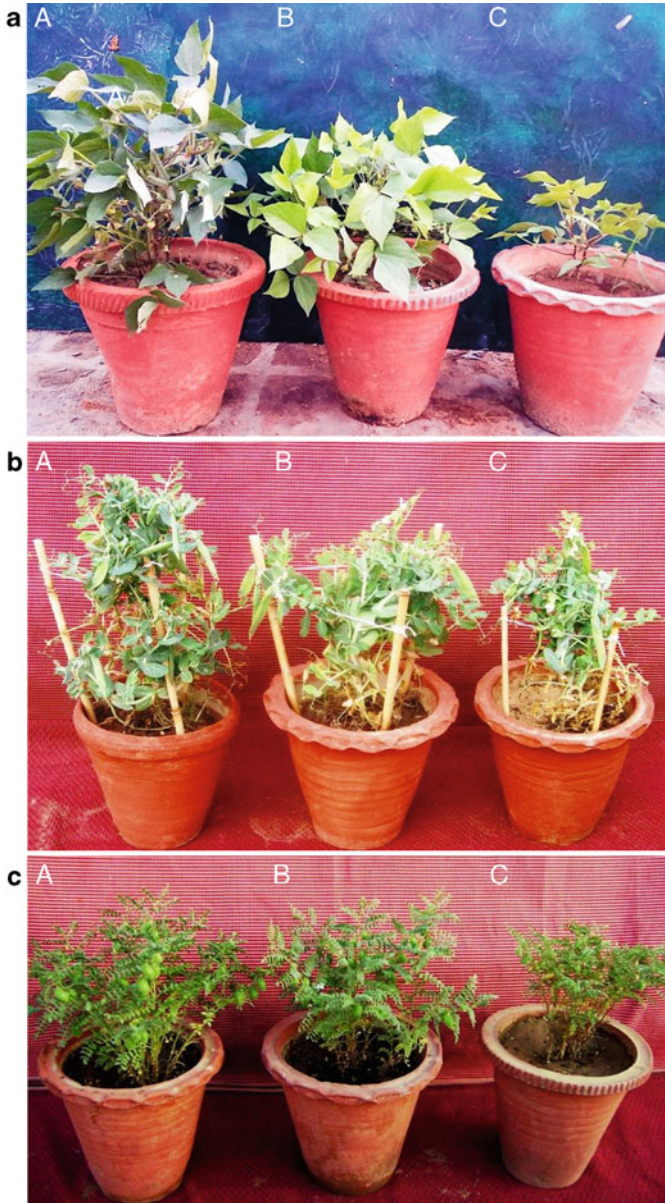
Fig. 2.1 (**a**) Greengram plants grown in sandy clay loam soil treated with *Bradyrhizobium* sp. (Vigna) alone (A), *Bradyrhizobium* sp. (Vigna) with cadmium (B) and cadmium alone (C) in a pot trial experiment. (**b**) Pea plants grown in sandy clay loam soil treated with *Rhizobium* alone (A), *Rhizobium* with copper (B) and copper alone (C) in a pot trial experiment. (**c**) Chickpea plants grown in sandy clay loam soil treated with *Mesorhizobium ciceri* alone (A), *Mesorhizobium ciceri* with chromium (B) and chromium alone (C) in a pot trial experiment
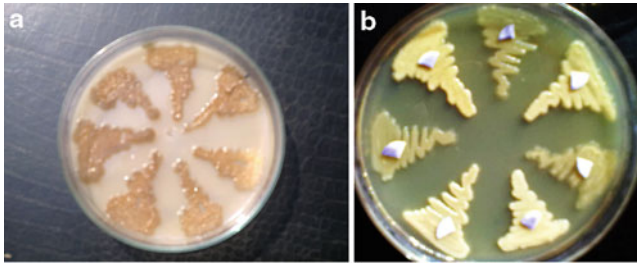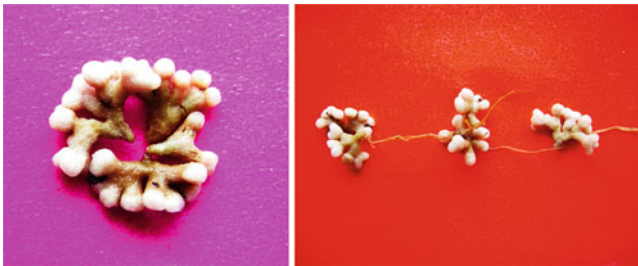
Plate 2.1 (**a**) *Azotobacter* and (**b**) Rhizobia

Fig. 2.2 Nodules morphology

Table 2.1 Current information on available rhizobial species

Genus	No. of species	Major host plants
Rhizobium	33	*Pisum*, *Phaseolus*, etc.
Sinorhizobium	12	*Acacia*, *Medicago*, etc.
Mesorhizobium	19	*Cicer*, *Prosopis*, etc.
Bradyrhizobium	08	*Glycine*, *Pachyrhizus*, etc.
Azorhizobium	02	*Sesbania*

(Compiled from: Rivas et al. (2009))

Medicago (Jordan and Allen 1974). The slow-growing species, *R. lupini* (Eckhardt et al. 1931), was found to nodulate *Lupinus* and *R. japonicum* (Buchanan 1926) mainly *Glycine max* (Jordan and Allen 1974). However, even after the role of Rhizobia was well established, this genus was less explored in terms of its diversity and functionality. Recently, with the advent of some newer molecular techniques and interest of rhizobiologist in exploring them as microbial inoculants for raising the productivity of crops especially legumes, the identification of rhizobial species from various hosts and locations has received a renewed attention. As a result, currently, Rhizobia have been reported to include more than 50 species (Table 2.1) distributed in genera *Rhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* (Velázquez et al. 2010). These rhizobial species carry symbiotic genes (located on plasmids or symbiotic islands) which codes for

nodulation and N_2 fixation. Interestingly, the host range of these genes can be extended to individual/groups of Rhizobia which do not have such genes. And therefore, upon acceptance by the recipient Rhizobia, such genes confer them the ability to nodulate legumes and fix atmospheric N.

2.3   *Rhizobium*–Legume Pairing: An Overview

Nitrogen is one of the prime elements required essentially for the synthesis of enzymes, proteins, chlorophyll, DNA and RNA. And hence, N plays a critical role in determining the health of living organisms including microbes and plants. For nodulating legumes, the N demand is fulfilled through symbiotic N_2 fixation (SNF) wherein atmospheric N_2 is converted to usable N (NH_3) by nitrogenase of Rhizobia (Shiferaw et al. 2004). The BNF accounts for about 65% of the total N currently utilized in agricultural practices which of course is believed to be continuously required in future sustainable crop production systems (Matiru and Dakora 2004). Rhizobial species of the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium* and *Sinorhizobium* intimately interact with legumes using flavonoid molecules as signal compounds, released by the legume host. These plant-generated compounds systematically induce the expression of nodulation (nod) genes in Rhizobia, which in turn produce lipo-chitooligosaccharide (LCO) signals called Nod factors (Perret et al. 2000; Shaw et al. 2006; Cooper 2007; Maj et al. 2010). These signal compounds trigger the mitotic cell division in roots, leading finally to nodule formation (Dakora 1995; Matiru and Dakora 2004; Jones et al. 2007; Batut et al. 2011). Inside the central nodule cells, Rhizobia are housed as symbiosomes that are horizontally acquired organelles and are involved in the enzymatic reduction of atmospheric N to NH_3 and make this N accessible to their hosts. In return, the Rhizobia get carbohydrates from their host. The host plants, however, regulate the number of nodules formed, the maturation of nodules and the N_2 fixation process.

2.4   How Rhizobia Promote Legume Growth?

Primarily, Rhizobia is known for its ability to provide N exclusively to legumes through BNF. However, the biologically available form of N produced by Rhizobia can also facilitate the overall growth of associated non-legumes directly by transferring symbiotically formed N to crops like cereals, growing in intercrops (Snapp et al. 1998) or to subsequent crops rotated with legumes (Hayat 2005; Hayat et al. 2008a, b). In addition to N_2 fixation, Rhizobia promote the growth of plants by other mechanism also (Table 2.2). For example, species of Rhizobia isolated from various sources such as conventional (Zaidi et al. 2003; Ahmad et al. 2008b; Ahemad and Khan 2011a) or stressed environment (Carrascoa et al. 2005; Wani et al. 2007c, 2008a) have shown the production of plant growth-promoting substances like phytohormones; auxins, cytokinins and abscisic acids; lumichrome, riboflavin, LCOs and vitamins (Keating et al. 1998; Wani et al. 2008c, 2009;

Table 2.2 Examples of plant growth-promoting substances synthesized by symbiotic nitrogen fixers

Symbiotic N$_2$ fixer	Crop enhancer	References
Bradyrhizobium MRM6	IAA, HCN, siderophore, ammonia, EPS	Ahemad and Khan (2011c)
Rhizobium MRL3	IAA, HCN, siderophore, ammonia	Ahemad and Khan (2011d)
Sinorhizobium strain	Chitinase	Qing-xia et al. (2011)
Rhizobium leguminosarum var. *phaseoli*	IAA	Stajkovic et al. (2011)
Rhizobium spp.	IAA, siderophore	Mehboob et al. (2011)
Sinorhizobium meliloti	IAA, P-solubilization	Bianco and Defez (2010)
Bradyrhizobium	IAA, gibberellic acid	Afzal et al. (2010)
Mesorhizobium	IAA	Ahemad and Khan (2010a)
Rhizobium spp.	IAA	Chakrabarti et al. (2010)
Rhizobium leguminosarum	IAA, siderophore	Ahemad and Khan (2010c)
Mesorhizobium	IAA, HCN, siderophore, ammonia, P-solubilization	Ahmad et al. (2008b)
Rhizobium strain TAL 1145	ACC-deaminase	Tittabutr et al. (2008)
Rhizobium spp.	IAA, gibberellic acid, zeatin	Boiero et al. (2007)
Mesorhizobium loti MP6	IAA, HCN, siderophore, P-solubilization	Chandra et al. (2007)
Rhizobium etli USDA9032	Phenazine, antibiotic	Krishnan et al. (2007)

Ahemad and Khan 2009, 2010b). Other plant growth-enhancing traits for which Rhizobia have been exploited includes synthesis of siderophore (Wani et al. 2008c; Ahemad and Khan 2011b), solubilization of inorganic P (Abd-Alla 1994; Chabot et al. 1996: Khan et al. 2007, 2009a, b, 2010) and as biocontrol agents (Khan et al. 2002; Deshwal et al. 2003a, b). Rhizobia isolated from nodules of some tropical legumes have also been shown to infect roots of crops other than legumes such as rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) via crack entry mechanism (Webster et al. 1997).

2.5   Heavy Metal Toxicity: A General Perspective

Heavy metals when present in lower concentration play an important role in the activities of many enzymes like proteinases, dehydrogenases and peptidases. Among metals, zinc, for example, is required in the synthesis of carbohydrates, proteins, phosphate, auxins, RNA and ribosome. Likewise, copper plays critical roles in various physiological processes such as respiration, photosynthesis, N and cell wall metabolism, carbohydrate distribution and seed production (Kabata-Pendias and Pendias 2001). However, in addition to some toxic metals, when the concentrations of even the biologically significant metals become higher, they cause toxicity. For example, cadmium even-though is not involved in any biological processes but may become quite toxic after it is accumulated inside the

organisms. Some of the nuisance of cadmium includes (1) disturbed enzyme activities, (2) inhibition of DNA-mediated transformation in microorganisms, (3) reduced symbiosis between microbes and plants and (4) increased plant predisposition to fungal invasion (Kabata-Pendias and Pendias 2001; Mohanpuria et al. 2007). In addition, stressors like heavy metal have also been reported to convert the viable bacterial cells to non-culturable form (Paton et al. 1997; Paudyal et al. 2007). Therefore, once the soil is destructed by heavy metals, metals found naturally within soil or accumulated as a result of anthropogenic activities (Giller et al. 1989; McGrath et al. 1995; Robinson et al. 2001; Lei et al. 2011), it becomes uninhabitable for microbial communities or unsuitable for crop production. For example, numerous metals (e.g. Cu, Ni. Zn, Cd, As) have been reported to inhibit the growth, morphology and activities of various groups of microorganisms (Khan and Scullion 2002; Shi et al. 2002; Lakzian et al. 2002; Bondarenko et al. 2010) including symbiotic N_2 fixers (McGrath et al. 1988; Santamaría 2003; Stan et al. 2011) like *R. leguminosarum*, *Mesorhizobium ciceri*, *Rhizobium* sp. and *Bradyrhizobium* sp. (Vigna) and *Sinorhizobium* (Wani 2008; Arora et al. 2010; Bianucci et al. 2011). On the other hand, Rhizobia among soil bacteria have been the organism of great interest for agronomist in general and legume growers in particular primarily due to their ability to provide N to plants. Considering the benefits of Rhizobia in N economy and the role of legumes in animal and human health, attention in recent times has been paid onto understanding how metals could affect the very survival of Rhizobia either present as free-living organism or when they are in intimate relationship (symbiosis) with legumes (Ibekwe et al. 1995; Khan et al. 2009b). Heavy metals are inhibitory to rhizosphere microorganisms, and processes mediated by them, like nitrogen-fixing ability of Rhizobia, are lost when they are in symbiotic association with the legume host, growing in metal-enriched locations (Vasseur et al. 1998; Barajas-Aceves and Dendooven 2001; Hernandez et al. 2003). For example, Arora et al. (2010) in a study assessed the impact of aluminium and copper, iron and molybdenum on growth and enzyme activity of fast- and slow-growing rhizobial species. Of the tested rhizobial strains, *Sinorhizobium meliloti* RMP$_5$ showed greatest tolerance to metal stress compared to *Bradyrhizobium* BMP$_1$. Both the strains were, however, extremely sensitive to Al than other metals. In addition, Al was found extremely toxic and reduced the various enzymatic activities like nitrate reduction, nitrite reduction and nitrogenase and hydrogenase uptake, by strains RMP$_5$ and BMP$_1$. Among the metals, copper had strong inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations. In comparison, all the tested enzymatic activities of *S. meliloti* RMP$_5$ increased up to the concentration of 0.1 mM Cu, while Fe enhanced the growth and enzyme activities of *S. meliloti* RMP$_5$ and *Bradyrhizobium* BMP$_1$ up to 100 mM concentration. Molybdenum increased all the tested enzymatic activities of *S. meliloti* RMP$_5$ up to 1 mM. Nitrate and nitrite reduction activities of *Bradyrhizobium* BMP$_1$ increased up to 1 mM concentration. However, nitrogenase and hydrogenase activities of *Bradyrhizobium* BMP$_1$ were enhanced only up to 0.5 mM Mo. In a similar study, Paudyal et al. (2007) determined the effect of three heavy metals (Al, Fe and Mo) on two strains of Rhizobia isolated from root nodules of two tropical legume species,

Mucuna pruriens and *Trigonella foenum-graecum*. All tested concentrations of aluminium had detrimental effect on rhizobial strains when grown in vitro and in vivo conditions. Iron, in contrast, supported bacterial growth and enhanced the symbiotic parameters such as biomass production and nodulation up to 25 μM which however had negative effect thereafter. Molybdenum at 75 μM improved bacterial growth, while up to 20 μM, molybdenum increased plant production and nodulation of test legumes. Hirsch et al. (1993), for example, demonstrated that the population of *R. leguminosarum* bv. *trifolii* was radically altered by long-term exposure to heavy metals, and this *Rhizobium* lost the ability to form functional symbiosis with white and red clover. In other study, Chaudri et al. (2000) in a long-term field trial reported a decrease in two agriculturally important species of Rhizobia, *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*, in soils, which were irrigated with sewage sludge containing Zn or Cu or mixture of Zn and Cu. Besides the potential toxicity of heavy metals on the growth and survival of Rhizobia, nodulation in legumes is also considerably affected (Khan et al. 2008). In sludge-treated soils, even though the nodulation on the root systems of clover was observed, the nodules were ineffective (McGrath et al. 1988; Giller et al. 1989). Similarly, Singh et al. (2003) noted that Pb reduced number and size of root hairs of greengram and also the darkness and total area of the leaves. Significant decrease in acetylene reduction by nodules or free-living heterotrophic nitrogen fixers in the presence of heavy metals has also been reported by others (Obbard et al. 1993; Shvaleva et al. 2010). McGrath et al. (1988) has shown a decrease in yield of white clover in monoculture on sludge-treated plots compared to plots receiving farm yard manure. If soils' heavy metal contents were at acceptable level after the amendment of the soil with sewage sludge, there was no negative effect on yield and N contents of alfalfa plants (Rebah et al. 2002).

2.5.1 Are Legumes Safe to Grow in Metal Contaminated Soils?

Legume–*Rhizobium* interactions occurring in either conventional or stressed environment have been one of the most widely studied and practical aspect in biological sciences. Rhizobia in general are used as inoculants for legume production in different agroecological environment and have shown a significantly higher pulse yields (Zaidi et al. 2003; Wani et al. 2007c; Ahemad and Khan 2011a). However, when grown in soils treated intentionally with heavy metals for experimental purpose or in soils already contaminated with heavy metals mainly due to contaminated agrochemicals and sewage sludge, most legume crops are not safe and affected negatively. The deleterious effects of heavy metals on nodulation and N_2 fixation of *Rhizobium*–legume symbiosis are probably due to their inhibitory effects on the growth and activity of both symbionts. For example, when 50–200 mg kg^{-1} soil of Co, Cu, Cd and Zn was added deliberately to soils used for *Lablab purpureus* cultivation, these metals invariably affected adversely the growth, nodulation and nitrogenase activity of plants in both pot and field trials. Apart from the effects of those tested metals on measured parameters, these metals also reduced substantially the level of nutrient elements like Na, K and Ca within

shoots of this plant which of course increased with increasing rates of metals applied (Younis 2007). Sepehri et al. (2006) in a greenhouse experiment showed that 2 mg Cd/kg soil had a variable effect on symbiotic properties of *S. meliloti* strains and consequently on *S. meliloti*–alfalfa symbiosis. A decreasing effect of cadmium concentration on root nodules and N concentration in plants inoculated with sensitive rhizobial strains in comparison with plants bacterized with tolerant strains was 68% and 41%, respectively.

Heavy metals when present in excess have also been found to delay the nodulation process in some legume crops. For example, with increasing concentration of arsenic (As) in the nutrient solution, there was greater time required for *Bradyrhizobium japonicum* strain CB1809 inoculated soybean (*Glycine max*) cv. Curringa plants to produce nodules, and the number of nodules per plant decreased at harvest. In addition, the inoculated plants had poor root hairs and dry matter contents in roots and shoots as the concentration in the solution increased (Reichman 2007). The abnormally higher concentrations of metal also limit the uptake of water and nutrients by plants (Terry 1981; Karpiscak et al. 2001) and concomitantly the health of plants. However, when a single or mixture of metals get a chance to enter within plant tissues and are translocated subsequently to various plant organs, they can interact directly with cellular components and disrupt the metabolic activities, causing cellular injuries and in some cases even may lead to the death of the plants (Fig. 2.3). As an example, cadmium even at considerably lower concentration was found toxic for the microsymbiont (Pereira et al. 2006; Younis 2007) and (1) inhibited the nitrogenase activity; (2) affected the plant biomass production; (3) disrupted nodule ultrastructure number of nodules and induced nodule senescence; (4) reduced dry matter accumulation in roots, shoot and leaf; and (5) adversely affected metabolic activities like photosynthesis of legumes (Balestrasse et al. 2004; Mumtaz et al. 2006; Wani et al. 2006; Noriega et al. 2007). Furthermore, cadmium-induced oxidative stress has led to the reduction in carbohydrate and protein (leghaemoglobin) synthesis within nodule and inhibited antioxidant enzyme activity. The increase in lipid peroxidation and thiols has also been found to result from cadmium toxicity for other crops (Balestrasse et al. 2003; Benavides et al. 2005; Garg and Aggarwal 2011). The increasing concentrations of heavy metals like cadmium, zinc and lead significantly decreased nodule index: the number of nodules per gramme of the total fresh biomass, at about 2.64 mg Cd kg^{-1}, 300 mg Zn kg^{-1} and 130 mg Pb kg^{-1}. From this study, it was proposed that the nodulation index of white clover could serve as a suitable bioindicator of increased heavy metal toxicity in soil (Manier et al. 2009). The effects of metals on rhizobial composition within soil or nodule environment and different legume genotypes, however, have been contradictory (Wani et al. 2007a, b, 2008a, b; Wani 2008). To validate this concept of conflicting effects of metals on Rhizobia, Paudyal et al. (2007) conducted an experiment which revealed that Rhizobia grew poorly in culture medium supplemented with even lower concentration of aluminium, while rhizobial growth was completely inhibited at 50 mM Al concentration (Wood and Cooper 1988; Chaudri et al. 1993; Broos et al. 2004). On the contrary, no reasonable changes in dynamics of *B. japonicum* and
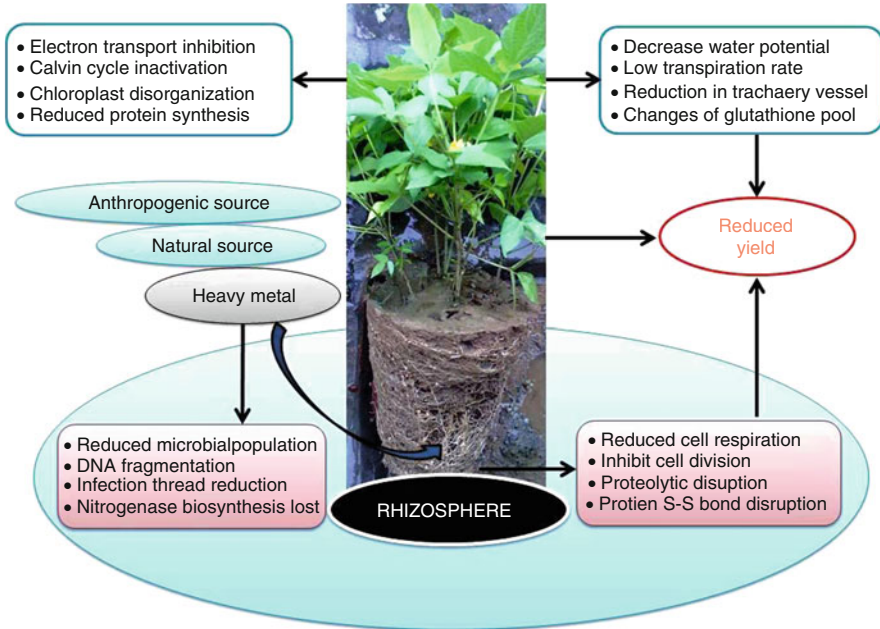
Fig. 2.3 Toxicity of heavy metal to various metabolic stages of plants including *Rhizobium*–legume symbiosis

growth and N_2 fixation by host plant, when grown in metal contaminated soils, were observed by others (Kinkle et al. 1987; El-Aziz et al. 1991; Smith and Giller 1992). Furthermore, it is suggested that there exist a relationship between *Rhizobium's* tolerance, heavy metal soil contamination and alterations in protein pool. Due to this, the assessment of variation in protein contents is considered a good indicator to estimate the level of stress imposed on *Rhizobium* populations exposed to heavy metal contamination (Pereira et al. 2006).

Conclusion

Heavy metal toxicity to some plants and microorganism is well documented, but its effect on legumes, *Rhizobium* and legume–*Rhizobium* symbiosis is poorly understood. These metals can arrest the growth and multiplication of Rhizobia in rhizosphere and may also have depressive effect on the steps involved in legume–*Rhizobium* symbiosis, resulting in low nitrogen fixation. In addition, metals can also cause severe toxicity to various metabolic activities of legumes including photosynthesis, synthesis of proteins, enzymes and carbohydrates. Therefore, understanding the metal–rhizobia–legume interaction in metal-enriched environment is urgently required for growing legumes in soils contaminated with heavy metals.

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Toxic Effects of Heavy Metals on Germination and Physiological Processes of Plants

3

Parvaze Ahmad Wani, Mohammad Saghir Khan, and Almas Zaidi

Abstract

Pollution of the environment by toxic metals in recent years has accelerated dramatically due to rapid industrial progress. Heavy metals when taken up in amounts in excess of the normal concentration produce lethal effects on plants, on microbes, and directly or indirectly on the human health. Deleterious impact of metals on plants includes the reduction in germinability of seeds, inactivation of enzymes, damage to cells by acting as antimetabolites, or formation of precipitates or chelates with essential metabolites. Heavy metals also show unconstructive effects on other physiological processes like photosynthesis, gaseous exchange, water relations, and mineral/nutrient absorption by plants. These adverse effects may be due to the generation of reactive oxygen species which may cause oxidative stress. The impact of heavy metals on germination of legume seeds and different physiological events of plants with special reference to leguminous plants grown in distinct agroecological niches is highlighted.

3.1 Introduction

Heavy metal pollution is one of the prime environmental problems that are caused due to the unabated, indiscriminate, and uncontrolled discharge of hazardous chemicals including heavy metals by different agencies into the environment (Fernandes and Henriques 1991; Cortes et al. 2003; Suciu et al. 2008; Navarro et al. 2008; Vaalgamaa and Conley 2008). Heavy metals released from such sources like metalliferous mining and smelting, use of chemicals in agricultural practices, and waste disposal and discharge of metals like cadmium, copper, chromium,

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mercury, nickel, lead, etc., from other sources, when build up in soils, can generate damaging effects on various agroecosystems (Mcllveen and Nagusanti 1994) including plants (Stimpfl et al. 2006; Pandey and Pandey 2009; Stobrawa and Lorenc-Plucińska 2008; Wani et al. 2008a, b) and on animals and human health (de Vries et al. 2007; Lagisz and Laskowski 2008; Korashy and El-Kadi 2008) via food chain. In general, it is reported that heavy metals unfavorably affect about 12% of the world's agricultural land (Moffat 1999). Even though some heavy metals like molybednium in low concentrations are required by plants to maintain its physiological functions (Hansch and Mendel 2009), the excessive accumulation of such metals in plant tissues can interfere and even disrupt the metabolic activities like photosynthetic and respiratory process, protein synthesis, and development of organelles of plants (Agarwala et al. 1995; Upadhyay and Panda 2009) including leguminous crops such as *Cajanus cajan* genotypes (Garg and Bhandari 2011). For example, two legumes like field pea (*Pisum sativum*) and fodder vetch (*Vicia villosa*) that were grown in field and glasshouse experiments with increasing concentrations multiple metals like Cd, Cr, Zn, Pb, Cu and Mn have been found more susceptible to soil metals (Wang et al. 2002).The dry matter yields of field pea and fodder vetch decreased by 169% and 113%, respectively, when grown in metal-contaminated soil. The concentration of copper was highest in fodder vetch, but the bioconcentration factors (BCF) of the metals declined with increasing soil metal loading rates except for chromium in fodder vetch (Wang et al. 2002). In a follow-up study, Wani et al. (2008a) evaluated the effects of cadmium, chromium, and copper by applying them, both singly and in combination, into soil where pea was grown as a test plant. Of the three metals, the sole application of copper had greatest toxicity against pea plants and depressed the seed yields by 15% at 1,338 mg Cu kg^{-1} relative to plants grown in untreated soils (control). Cadmium and chromium, in contrast, showed a variable increase in the measured parameters. Moreover, the metal uptake by roots and shoots quantified in 90-day-old plants and in grains of pea plants measured at 120 days after sowing varied among treatments of metals. On the other hand, there are few reports regarding rhizobia–legume association which has been found insensitive to metals and could even increase N concentration in metal-contaminated soils. For example, the symbiotic association between the legume *Anthyllis vulneraria* subsp. *carpatica* and the bacterium *Mesorhizobium metallidurans* isolated from highly polluted mine tailings significantly increased N pool of soils heavily contaminated with zinc, lead, and cadmium (Mahieu et al. 2011). Of the total soil N pool, about 80% N was due to biological nitrogen fixation (BNF) resulting from metallicolous *A. vulneraria* and the rhizobial interaction happening in metal-enriched soil. This finding suggests that *A. vulneraria* due to its ability to establish a functional symbiosis even in metal-enriched soils could be used to facilitate a low-maintenance plant cover besides stabilizing the vegetation in high heavy-metal-contaminated soils.

Mechanistically, the toxic effects of metals on plants include the inactivation of enzymes, impairment of membrane function and loss of membrane integrity, deranged nutrient absorption, cell damage, or precipitate or chelate formation with essential plant metabolites. The threat by heavy metals to plants and consequently to humans and other animals is further provoked by poor movement and

their ability to persist in the environment. For instance, lead may persist in soil for about 150–5,000 years, while the average biological half-life of cadmium has been reported as 18 years (Forstner 1995). Apart from the effects of heavy metals on plants, numerous studies have also shown that long-term accumulation of heavy metal in soils deleteriously affects community diversity including those of denitrifying community (Sobolev and Begonia 2008) or soil microbial activities (Doelman and Haanstra 1984; Hemida et al. 1997; Solanki and Dhankhar 2011) including those of hydrogenase activity of *Rhizobium leguminosarum* biovar viciae (Ureta et al. 2005). For example, in a study, the impact of aluminum and heavy metals such as copper, iron, and molybdenum on growth and activity of enzymes of fast- and slow-growing rhizobial species was determined (Arora et al. 2010). Of these, copper had inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations. However, enzymatic activities in *Sinorhizobium meliloti* RMP$_5$ increased up to the concentration of 0.1 mM copper. Iron on the other hand enhanced the growth and enzyme activities of *S. meliloti* RMP$_5$ and *Bradyrhizobium* BMP$_1$ up to 100 mM concentration. Molybdenum augmented the enzymatic activities of *S. meliloti* RMP$_5$ up to 1 mM. Nitrate and nitrite reduction activities of *Bradyrhizobium* BMP$_1$ were amplified up to 1 mM concentration. Nitrogenase and hydrogenase activities of *Bradyrhizobium* BMP$_1$ were accelerated only up to 0.5 mM Mo. Therefore, the toxicity of heavy metals to various life forms has attracted considerable research attention in recent years because of the regular and unrestrained release of pollutants into a variety of agroecosystems.

3.2   Metal Uptake, Translocation, and Accumulation

Heavy metals are defined as groups of elements that have specific weights of higher than 5 g/cm^3 (Holleman and Wiberg 1985). After heavy metals are deposited into soils from various sources (Giller et al. 1989; McGrath et al. 1995), they first interact with root systems of plants and are absorbed via uptake mechanisms (Seuntjens et al. 2004). The degree to which higher plants can take up metal depends on their concentration in soil and availability to plants. The uptake of metals by plant roots depends on (1) diffusion of elements along the concentration gradient, (2) root interception, where soil volume is displaced by root volume due to root growth, and (3) mass flow, transport from bulk soil solution along the water potential gradient. Some metals in plants can be absorbed by the apical region, while others are taken up by the entire root surface. Thereafter, metal is transported further into the cells, some to the apoplast, and some are bound to cell wall substances. From apoplast, metals further migrate through the plasma membrane into the cytoplasm where metal affects the nutrient status of the plants. For instance, the toxic effects of chromium are due to its speciation, which determines its uptake, translocation, and accumulation. Uptake and accumulation of chromium or copper by various crops are well documented (Peralta et al. 2001; Shanker et al. 2003; Cambrolle et al. 2011). When uptake by the root is high and the nutrient

concentration in the soil is low, element uptake is limited by diffusion. Since there are some essential metals, at least the uptake of these ought to be regulated. Zinc is transported with Zn transporters, with a higher abundance in Zn accumulator species than in nonaccumulator species (Lasat et al. 2000). Zinc is also known to be actively transported as a free ion across the tonoplast. Other metals (e.g., cadmium) easily enter the root through the cortical tissue and are translocated to the above-ground tissues (Yang et al. 1998). As soon as metals, for example, cadmium, enter the roots, they reach the xylem through an apoplastic or symplastic pathway (Salt et al. 1995a) and form a complex with ligands, such as organic acids and/or phytochelatins (Salt et al. 1995b). Normally, cadmium ions are retained in the roots, and only very small amounts are transported to the shoots. Metal ions are probably taken up into cells by membrane transport proteins designed for acquisition of nutrient metals. In a study, Cd and Zn have been found to coexist in aerial parts of *Arabidopsis halleri* (Bert et al. 2003) plants, suggesting that Cd and Zn uptake are genetically correlated and that these metals are taken up by the same transporters or that their transporters, when different, are controlled by common regulators.

3.3 Toxicity of Heavy Metals to Plants

Plants respond differently to different heavy metals present in the soil. The toxicity of heavy metals varies with the genotypes, age and developmental stages of plants (Shaw and Rout 2002) and is influenced by the physicochemical properties of the soil, root exudates, and concentration of metals in the soils. Moreover, differences in solubility, absorbability, transport, and chemical reactivity of metals also lead to variation in toxicity to plants (Stohs and Bagchi 1995). The elevated concentrations of heavy metals in agronomic soils, however, result in mineral deficiency (Gonçalves et al. 2009; Bouazizi et al. 2010; Lequeux et al. 2010), disturb the nutrient uptake and nutrient status, for example, as observed in roots and leaves of faba bean (Moussa 2004) and green gram [*Vigna radiata* (L.) Wilczek] (Manivasa-gaperumal et al. 2011), and hence adversely affect different metabolic activities of plants including cellular redox homeostasis and cell necrosis, as observed in alfalfa (*Medicago sativa*) (Ortega-Villasante et al. 2005), leading thereby to a decrease in overall growth of plants (Panda and Choudhary 2005). Toxicity may result from the binding of metals to sulfhydryl groups of proteins, leading to an inhibition of activity or disruption of protein structure (Das et al. 1997) or enzyme activity (Tyler et al. 1989; Arun et al. 2005). In addition, the elevated concentrations of metals may stimulate the formation of free radicals and reactive oxygen species (ROS) (Fornazier et al. 2002).

3.3.1 How Heavy Metals Act

Before understanding how heavy metals exhibit toxicity, one needs to take into consideration the chemical properties of such toxic elements. Most of the heavy

metals are transition metals having an incompletely filled δ-orbital present at cations at physiological conditions. The aerobic cells have physiological redox between −420 mV and +800 mV. Therefore, heavy metals can be divided into redox active and inactive metals. Metals with lower redox potential than those of biological molecules cannot participate in biological redox reactions. One of the mechanisms causing toxicity involves autoxidation of redox active metals such as Fe^{2+} or Cu^{2+} in O_2^- formation and subsequently to H_2O_2 and OH^- via Fenton-type reactions. Cellular injury by this type of mechanism is well demonstrated for iron (Imlay et al. 1988), copper (Li and Trush 1993a, b), and for other metals (Lund et al. 1993). Another mechanism of heavy metal toxicity is their ability to bind strongly with oxygen, nitrogen, and sulfur atoms (Nieboer and Richardson 1980). This binding affinity is related to free enthalpy of the formation of the product of metal and ligand. Thus, due to these activities, heavy metals can inactivate the enzymes by binding to cysteine residues.

3.3.2 Seed Germination and Physiological Processes Affected by Heavy Metals

Heavy metals at high concentrations inhibit various stages of plants starting with seed germination to the growth and development of plants by disturbing many biochemical and physiological processes, such as membrane destruction, transpiration reduction, protein synthesis impairment, photosynthetic apparatus distortion, enzymes inactivation, and lipid peroxidation (Sanita di Toppi and Gabbrielli 1999; Talanova et al. 2000; Monni et al. 2001; Parmar and Chanda 2005; Seregin and Kozhevnikova 2006; Vijayaragavan et al. 2011). Effects of different heavy metals on legume seed germination are discussed in the following section.

3.3.2.1 Legume Germination Under Metal Stress

Seed germination in general is an intricate process which begins with the imbibition of water. Thereafter, it is regulated by hormonal interactions (endogenous) and environmental (exogenous) factors. Among fundamental factors, the seed embryo serves as a good source of gibberellic acid, which plays an important role in the germination of seeds and establishment of seedlings. In addition, gibberellin affects leaf expansion, stem elongation, flower initiation, and flower and fruit development (Salisbury and Ross 1992; Dewar et al. 1998; Hamman et al. 2003). Other hormones that affect growth and development of plants include abscisic acid (Monni et al. 2001; Pospíšilová 2003), cytokinins (Van Staden et al. 1982; Letham and Palni 1983; Letham 1994), and zeatin and zeatin riboside (Atici et al. 2005). Even though seed germination is one of the most important and first critical phase in the life cycle of plants, practically little information is available on how heavy metals affect the germinating seeds. While seed germination is considered a sensitive process compared to other stages of plant development (Ernst 1998) and seed coat may be impermeable to heavy metals, considerable amounts of metals are accumulated within legume seeds (Gross et al. 1987). As a result, all biologically

relevant macromolecules, like nucleic acids, membrane lipids, and proteins, have been found susceptible to damage by ROS. Accordingly, numerous studies have reported the production of ROS during the germination of various species (Bailly 2004), and the production of ROS by germinating seeds has been due to certain stressors like heavy metals. This in turn affects the success of germination. The effects of heavy metals on seed germinations, however, have been conflicting (Hsu and Chou 1992; Atıcı et al. 2003). For example, varying concentrations of aluminum and chromium when used either alone or in combination had no reductive effect on percentage germination of legumes such as *Vigna radiata* and *V. sinensis* (Jamal et al. 2006). In contrast, different concentrations of cadmium chloride (CdCl$_2$) decreased the germination percentage and the germination rate index (GRI) of cowpea (*Vigna unguiculata* L.) (AI-Rumaih et al. 2001). Similarly, the inhibitory and toxic effects of lead on the germination of seeds of *Lupinus luteus* (Wozny et al. 1982) and pea (*Pisum sativum*) (Wierzbicka and Obidzinska 1998) and cadmium on pea (Smiri 2011) are reported. In yet another study, Atici et al. (2005) observed that lead and zinc significantly delayed and impeded the germination of chickpea (*Cicer arietinum* cv. Aziziye-94) seeds. The negative effect of lead on germination was higher than that of zinc. In a recent study, Talukdar (2011) evaluated the effect of five different concentrations (0, 10, 20, 30, and 40 mg/L) of arsenic on two important leguminous crops, namely, *Trigonella foenum-graecum* L. (fenugreek) and *Lathyrus sativus* L. (grass pea), especially germination and early seedling growth stage. The germination percentage, germination index, and relative germination rate in both plants decreased with consistent increase in arsenic level, and the effect was maximum at 30 and 40 mg/L.

In addition to the direct effect of metals on germinating efficiency of seeds, it is also important to understand how heavy metals affect the level of endogenous chemical compounds including hormones in plants growing under stressed environments. In this context, Atici et al. (2005) while evaluating metal toxicity found that lead increased the abscisic acid (ABA) and zeatin (Z) contents while it decreased gibberellic acid (GA3) content in the germinating chickpea seeds. The high concentrations of zinc (1 and 10 mM) decreased contents of Z, zeatin riboside (ZR), and GA3 while 0.1 mM zinc increased the content of the same hormones. The ABA content was enhanced by zinc at all tested concentrations. A negative correlation has also been observed between gibberellic acid and cytokinins in chickpea seeds germinating at low cadmium concentrations but not at high concentrations (Atıcı et al. 2003). Similar reports on the inhibitory and toxic effects of lead on seed germination and endogenous chemical compounds of crops other than legumes such as *Oryza sativa* (Hsu and Kao 2003; Jayakumar et al. 2008), *Sinapis alba* (Fargasova 1994), *Sonchus oleraceus* (Xiong 1997), and *Brassica pekinensis* (Xiong 1998) are available in the literature. The reduction in seed germination or level of endogenous compounds in seeds when allowed to grow in contaminated environment has been suggested due to different reasons. For example, the decrease in the germination percentage of plants such as cowpea seeds may be related to the negative effects of metals like cadmium on water uptake and water movement (Poschenreider et al. 1989; Vassilev et al. 1998). In addition,

Barcelo et al. (1986) indicated that cadmium affected water relations by decreasing both water absorption and transport and also by lowering water stress tolerance. Therefore, the higher cadmium concentration in the germination medium of cowpea seeds seems to reduce the availability of water in the embryo axis, and this may be the reason for the low seedling establishment. Considering these and other associated data, it is concluded that the hormonal response of germinating seeds including those of legumes to different heavy metals (essential or nonessential for plant) be evaluated in order to improve the quality of plants.

3.3.2.2 Physiological Processes Affected by Heavy Metals
Cell Wall and Plasma Membrane

Roots of the various plants including legumes are the first organ that is directly exposed to metals in soils and hence are the target of stressor molecules including heavy metals. After uptake by plants, heavy metals interact very strongly with the plant cell wall (Ernst et al. 1990), but the binding properties and its role in the mechanism of metal tolerance have been controversial (Verkleij and Schat 1990). Most of the heavy metals bind to polygalacturonic acids, to which the affinity of metal ions vary considerably (Ernst et al. 1992). Damage of the cell membrane system particularly the plasma membrane is the other target site of heavy metal toxicity (Chaoui et al. 1997; Janicka-Russak et al. 2008). Upon interaction, metal induces changes in membrane lipids both qualitatively and quantitatively which in turn alter the structure and permeability of membrane leading to ion leakage (Ouzounidou et al. 1992; Berglund et al. 2002; Bouazizi et al. 2010) and other cellular processes. For example, oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as H^+ ATPase in roots of cucumber (*Cucumis sativus*), or changes in the composition and fluidity of membrane lipids are some of the toxicity consequences of metals (Mehrag 1993; Janicka-Russak et al. 2008). Among different metals, the effect of chromium on the transport activities of plasma membrane is reported by Zaccheo et al. (1982). The inhibition of ATPase activity is suggested to be due to the disruption of the membrane by free radical generated under metal stress. The decrease in ATPase activity declines proton extrusion and ultimately reduces the transport activities of the root plasma membrane. As a result, the uptake of nutrients by roots is inhibited. The primary toxicity effect leading to membrane damage, due to the high oxidation power of Cr (VI), for example, has been observed in bush bean (*Phaseolus vulgaris* L.) grown on perlite treated with hexavalent chromium salt (Na_2CrO_4). It was further suggested that chromium was retained in vacuoles and cell walls of roots and that the chromium reaching the leaves may be principally Cr III and present in cell walls (Vazques et al. 1987). Moreover, it is also reported that chromium interferes with the mechanism controlling intracellular pH (Zaccheo et al. 1985). Generally, the chromium stress can induce the following metabolic modification in plants: (1) alteration in the production of pigments like chlorophyll and (2) increased production of metabolites, for example, glutathione and ascorbic acid, as a direct response to metal stress which may cause damage to the plants. Among other metals, cadmium and copper have also been found to adversely affect the lipid

composition of membranes (Quartacci et al. 2001). Moreover, cadmium treatment has also been shown to reduce ATPase activity of the plasma membrane fraction of roots (Fodor et al. 1995). Considering all these events together, it may be concluded that heavy metals increase the permeability of membrane in a nonspecific manner along with considerable decrease in specific transporting activities which is likely to disrupt the ionic homeostasis. Consequently, the activities of many enzymes important for basic cell functions are disrupted.

Lipid Peroxidation

In addition to the metal-induced changes in fatty acid composition of membranes, membrane injury is also related often to an increased peroxidation of membrane lipids, resulting from the action of highly toxic free radicals. In this context, several metal ions have been reported to cause peroxidation of lipids of both the plasma membrane and chloroplast membrane (Hernandez and Cooke 1997). As a result of this activity, the synthesis of ROS increases. For example, certain metals such as cadmium, copper, zinc, nickel, chromium, etc., have been reported to enhance lipid peroxidation (Chaoui et al. 1997; Hartley-Whitaker et al. 2001). In general, iron and copper compounds are known to generate more free radicals than other metals and increase the peroxidation (Price and Hendry 1991). For example, lipid peroxidation after copper treatment was found highest in root tissues of bean plants (Yurekli and Porgali 2006). However, in other plant organs like stem and leaf tissues, no significant increase in lipid peroxidation was observed. Both lipid peroxidation and excessive copper accumulation in root tissue were suggestive of the fact that oxidative stress and lipid peroxidation were due to the release of free radicals in the root tissues of bean plants (Yurekli and Porgali 2006). In contrast, the level of lipid peroxidation quantified as MDA content though decreased in roots but increased in leaves of *Vigna mungo* (L.) grown in perlite–vermiculite using Hoagland nutrient solution treated with 40 μM cadmium (Molina et al. 2008). In line with these findings, other researchers have also reported that copper is capable of forming toxic oxygen and starting the process of lipid peroxidation quite effectively (Girotti 1985; Luna et al. 1994; Weckx and Clijsters 1996). Thus, the variation in membrane functions caused by metals could be due to changes both in the structure and peroxidation of membrane lipids (Cakmak and Horst 1991). As an example, aluminum has been reported to cause lipid peroxidation by disorganizing the membrane structure by generating free radicals (Weckx and Clijsters 1996). The increased lipid peroxidation also changes membrane properties, such as fluidity and permeability, and modulates the activities of membrane-bound ATPases (Shewfelt and Erickson 1991). Indeed, peroxidation is a chain reaction in which unsaturated fatty acids are converted stepwise into various small hydrocarbon fragments, such as malondialdehyde (Kappus 1985). The lipid peroxidation processes and the resulting substances in turn severely affect the functioning of the plasma membrane leading ultimately to the death of the cells. For example, Zhang et al. (2007) found that the different concentration of heavy metals like Pb^{2+}, Cd^{2+}, and Hg^{2+} enhanced the lipid peroxidation of *Bruguiera gymnorrhiza* plants when this plant was grown under the stress of these metals. Similarly, Nasim and Dhir (2010) also found that

the heavy metals resulted in the lipid peroxidation of the cell membrane of the medicinal plants exposed to heavy metals.

Photosynthesis

Photosynthesis is other important physiological event of plants which is reported to be adversely affected by heavy metals when plants are grown in metal-enriched soils (Thomas et al. 1998; Monni et al. 2000; Zeid 2001; Sharma and Sharma 2003; Molina et al. 2008; Mateos-Naranjo et al. 2008). For example, even though the high concentration of both lead and chromium in the rooting media drastically reduced the photosynthetic pigments of mash bean [*Vigna mungo* (L.) Hepper] cultivar FS-1 and mash-97, chromium application had more damaging effect as compared to lead (Hussain et al. 2006). The decreased supply of photosynthates to the actively growing organs consequently diminishes the plant growth (Fargašová 1998). Moreover, the poor vegetative growth under metal stress also inhibits reproductive growth of plants (Arun et al. 2005). It is, however, generally believed that the toxic metals react with the photosynthetic apparatus at various levels of organization and architecture resulting in (1) accumulation of metals in leaves; (2) alteration in the functions of chloroplast membrane and partitioning in leaf tissues like stomata, mesophyll, and bundle sheath; (3) reduction in photosynthetic efficiency ($^{14}CO_2$ fixation), ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCo) activity, and leaf pigment content (Moussa 2004); (4) metal interaction with cytosolic enzymes and organics; (5) supramolecular level action particularly on photosystem I, photosystem II, membrane acyl liquids, and carrier proteins in vascular tissues; and (6) molecular level interactions, particularly with photosynthetic carbon reduction (PCR) cycle enzymes, xanthophylls cycle, and adenylates. Like many other crops, the photosynthetic pigments and the photosynthetic process, like those involved in the reduction of carbon, have been found to be negatively affected when legumes are grown in heavy-metal-contaminated soils (Bibi and Hussain 2005). For example, excess concentrations of copper modified the ultrastructure of chloroplast in runner beans (*Phaseolus coccineus* L.) (Maksymiec et al. 1995) while reduction in photosynthetic pigments like chlorophyll of other legumes grown in metal-treated soils is reported by Mysliwa-Kurdziel and Strzatka (2002). Of the different photosynthetic components, both chlorophyll a and b have been found highly sensitive to varying levels of metals like lead and chromium in mash bean cultivars (Hussain et al. 2006; Gajewska et al. 2006) or due to copper in bean plants (Yurekli and Porgali 2006). The decrease in the chlorophyll a/b ratio following chromium application, for example, has been suggested due to the destabilization and degradation of the proteins of the peripheral part (Shanker 2003). In contrast, the reduced supply of both copper and molybdenum has also shown reduction in chlorophyll a and b and carotenoids in pea plants grown at full-strength Helriegel nutrient solution competed with micronutrients as in Hoagland and Arnon and reduced supply of Mo and Cu (Hristozkova et al. 2006). The inactivation of enzymes involved in the chlorophyll biosynthetic pathway could thus contribute to the general reduction in chlorophyll content in most plants including legumes under heavy metal stress. However, the majority of reports

on the impact of heavy metals on photosystem II activity have been observed for Cd^{2+} and Cu^{2+}, whereas Cd^{2+} affects both the PS II reaction center and the light-harvesting complex (LHC) and causes an inefficient energy transfer from the LHC to the reaction center. In addition, enzymes of the PCR cycle are inhibited under heavy metal stress while the key steps of the Calvin cycle, like carboxylation, reduction, and regeneration, have also been found to be affected by heavy metals. Of these, carboxylation is the most sensitive stage for metal toxicity. Among various metals, cadmium exerts its toxicity by damaging membrane and inactivating enzymes, possibly interacting with sulfhydryl groups of proteins (Fuhrer 1988) as also reported for Pb^{2+}, Cd^{2+}, Zn^{2+}, and Cu^{2+} (Van Assche and Clijsters 1990). In some cases, heavy metal toxicity is, however, reflected by an increase in the activity of these enzymes, for instance, malic enzyme, glucose-6-phosphate dehydrogenase, and peroxidase in leaves. Cadmium has been the most intensively studied inhibitor of dark reactions of photosynthesis (Krupa 1999). It was shown in isolated protoplasts treated with Cd^{2+} that the main target of this metal action was the reactions of the Calvin cycle and that activation of RuBisCo was not affected (Weigel 1985). In contrast, Sheoran et al. (1990) showed significant reduction of RuBisCo activity of pigeon pea (*Cajanus cajan* L.) plants, treated with Cd^{2+} at an early growth stage. However, in older plants, the activity of RuBisCo was not affected. They concluded that the reduction in photosynthesis was due to decrease in chlorophyll content, effects on stomatal conductance, and the electron transport system.

Water Relations

Wilting of various crops and plant species due to metal toxicity has been reported, but little information is available on the exact effect of metal on water relations of higher plants. However, Barcelo et al. (1985) observed a decrease in leaf water potential in chromium-treated bean plants. Excess concentration of chromium though decreased the water potential and transpiration rates, yet it increased the diffusive resistance and relative water content of cauliflower (*Brassica oleracea*) leaves (Chatterjee and Chatterjee 2000). Similarly, decreased turgor and plasmolysis was observed in epidermal and cortical cells of bush bean (*Phaseolus vulgaris* L.) plants grown on perlite exposed to chromium (VI) used as Na_2CrO_4 (Vazques et al. 1987). Toxic levels of chromium in beans were found to decrease tracheary vessel diameter, thereby reducing longitudinal water movement. Impaired spatial distribution and reduced root surface of Cr-stressed plants can lower the capacity of plants to explore the soil surface for water. The significantly higher toxic effect of Cr(VI) in declining the stomatal conductance could be due to the high oxidative potential of Cr(VI), which in turn may be instrumental in damaging the cells and membrane of stomatal guard cells. Some of the effects of chromium on various physiological processes of different crops are listed in Table 3.1.

Nitrate Reductase

Nitrate reductase (NR) is one of the principal enzymes involved in nitrate assimilation process in higher plants and catalyzes the first step of nitrate assimilation pathway.

Table 3.1 Effect of chromium on plant functions

Process	Crop/plant	Physiological effects
Photosynthesis	Wheat, peas, rice, maize, beans, sunflower	Electron transport inhibition, Calvin cycle enzyme inactivation, reduced CO_2 fixation, chloroplast disorganization
Water relations	Bush beans, sunflower, mung bean	Decreased water potential, increased transpiration rate, reduced diffusive resistance, wilting, reduction in tracheary vessel diameter
Mineral nutrition	Soybean, tomato, bush bean, sunflower, maize	Uptake of N, P, K, Fe, Mg, Mn, Mo, Zn, Cu, Ca, B affected
Enzymes and other compounds	*Nymphaea alba* and various cereals and legumes	Inhibition of assimilatory enzymes, increased activity of ROS-scavenging enzymes, changes in glutathione pool, no production of phytochelatins

However, like other plant physiological functions, NR is also repressed by heavy metals present in the environment. For example, when *Phaseolus vulgaris*, an important leguminous crop and a major source of nutrition to millions of people, was grown with mercury, it showed concentration-dependent response for NR activity. When glutathione (GSH) and cysteine (Cys) were also used either alone or in combination with mercury, there was a considerable increase in NR activity relative to sole application of mercury. However, the addition of buthionine sulfoximine (BSO) to Hg and GSH/Cys resulted in the reversal of the toxic effect of mercury against glutathione or cysteine. The protective effect of glutathione was, however, more profound in comparison to cysteine. From this study, two important findings were clear: (1) that the enzyme inactivation was due to binding of mercury with thiol ($-SH$) groups of protein and (2) that glutathione served as precursor for phytochelatins (Sharma and Subhadra 2010). In another study by Shalaby and Al-Wakeel (1995), nodules collected from faba bean (*Vicia faba* L. cv. Giza 3) grown for 90 days in a clay–loam-soil-containing pots had a vigorous NR activity whereas there was no detectable activity in leaves. The NR activity of the nodule was significantly decreased when plant was sprayed with increasing concentrations (0–1,000 μM) of Al^{3+} or Cd^{2+}. This reduction in NR activity was more obvious following cadmium treatment, and the specific activity of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) was more visible in the 60-day-old plants compared to those observed for 90-day-old plants. Furthermore, the GOT activity was consistently greater than GPT, but GOT was more sensitive to Al^{3+} and Cd^{2+} application, and a concentration-dependent reduction in its activity was recorded. Of the two metals, Cd^{2+} had higher toxic effect than Al^{3+} on nodule GOT activity. Interestingly, it is not only the higher concentration of metals that reduces the NR activity, but there are certain metals like the molybdenum and copper, whose reduced supply could also limit the activities of the enzymes (nitrate reductase and glutamine synthetase) and fresh weight of legumes, for example, pea plants (Hristozkova et al. 2006). Accumulation of nitrates in plant tissues was however enhanced, especially in the pea variants with restrictive copper concentration.

Apart from legumes, metals like nickel have also shown inhibitory effect on the NR activity of other crops such as New Zealand spinach (*Tetragonia expansa* Murr.) and lettuce (*Lactuca sativa* L. cv. Justyna) plants grown with different N forms and regimes (Matraszek 2008). As an example, nickel at 0.4 μM although did not cause any noteworthy change in the NR activity in lettuce plants supplied with nitrate alone or mixture of nitrate and NH_4NO_3, NR activity in New Zealand spinach leaves was decreased (in the presence of nitrate) and increased following combined application of nitrate and NH_4NO_3. In contrast, nickel at 40 or 80 μM significantly decreased the NR activity in New Zealand spinach plants treated with nitrate or mixed N forms. The reduction in NR activity was, however, more prominent in leaves than in roots. No significant change in the NR activity was, however, recorded in spinach leaves when plants were grown with 40 μM Ni and mixed N. In general, the NR activity was significantly dropped in the above-ground parts of nickel-stressed lettuce plants supplied with NO_3^-–N or NH_4NO_3. On the other hand, no major change was noticed in lettuce roots, except for a decline in the NR activity in the roots of NO_3^--fed plants grown in the nutrient solution containing 80 μM Ni. Further addition of nickel, however, did not affect the NR activity in New Zealand spinach plants but increased it in lettuce organs, especially in roots. In a similar study, NR activity of leaves was significantly increased over control values and negatively correlated with root and shoot length, leaf area, and biomass of the plants, indicating stress due to Cr(VI) in *A. Lebbeck* (Tripathi et al. 1999). Chromium concentrations up to 200 μM resulted in a significant inhibition of NR activity in *Nelumbo nucifera* (Vajpayee et al. 1999) and *Nymphaea alba* (Vajpayee et al. 2000). Seedlings treated with 1 μM chromium increased NR activity, whereas higher chromium concentrations were toxic and reduced the enzyme activity significantly in wheat (Panda and Patra 2000).

Denitrification Activity

Denitrification is a natural microbial process where nitrate is changed to dinitrogen gas during anaerobic respiration. Such reduction occurs sequentially, during which nitrate is converted to nitrite, nitric oxide, nitrous oxide, and, finally, nitrogen gas. Denitrification involves several proteins that require metal ions as a cofactor (Ferguson 1998; Philippot and Højberg 1999). Molybdenum, for example, acts as a component of the molybdenum cofactor of nitrate reductase, while iron is required for the cytochrome subunits of both nitrate and nitrite reductases. A high dosage of trace metals, such as Fe, Cu, Zn, Mo, and Mn, is known to increase denitrification rate. For example, Cyplik et al. (2007) reported that an addition of Fe, Cu, and Mo significantly increased the specific nitrate reduction rate of *Haloferax denitrificans*. If affected by heavy metals, altered denitrification could lead to a number of undesirable effects including the effect on human health. However, fewer assessments have been made on denitrification (Sakadevan et al. 1999; Holtan-Hartwig et al. 2002). Even if it occurs, suppressed denitrification in soil is likely to lead to enhanced N 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 which depend on surface waters for municipal, recreational, or agricultural purposes. Nitrous oxide reductase affected by metals has been observed (Holtan-Hartwig et al. 2002) to cause incomplete denitrification leading to emission of nitrous (and possibly nitric) oxides. Nitrous oxide is a potent greenhouse gas that damages ozone layer (Dickinson and Cicerone 1986). Moreover, denitrification disruption via metal contamination could act as a link between local metal contamination and global climate change phenomena. While assessing the impact of various metals on denitrification process, Yang et al. (2005) found that cadmium had the most toxic effect and inhibited denitrification effectively while denitrification of maize (*Zea mays*) was reduced by the application of other heavy metals (Yangye 2005).

Antioxidant Defenses
The exposure of plants to excess concentration of heavy metals such as iron, copper, zinc, nickel, manganese, and lead results in oxidative injury (Mazhoudi et al. 1997) eliciting enzymatic and nonenzymatic antioxidative reaction responses and lipid peroxidation. The ROS so generated may, however, damage carbohydrates and complex molecules, lipids, nucleic acids, proteins, and amino acids produced by the cells. Cells on the other hand have evolved certain strategies like enzymatic antioxidant systems for synthesis of enzymes such as superoxide dismutase+ (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POD: EC 1.11.1.7), and nonenzymatic antioxidants like ascorbate, glutathione (GSH), and phenolic compounds to combat oxidative stress. The antioxidant protection to relieve the heavy metal stress by plants appears to be limited since increased concentration of pollutants decreases the activity of antioxidant enzymes like glutathione reductase (GR) and catalase (CAT) as observed in the leaves of faba bean plants grown in the presence of $CdCl_2$ (Moussa 2004). A similar higher malondialdehyde content and SOD and POD activities have been detected when soybean (*Glycine max*) plants were exposed to the mixture of aluminum and cadmium (Shamsi et al. 2008). Reddy et al. (2005) in a study exposed 1-month-old horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. cv. VZM1] and Bengal gram (*Cicer arietinum* L. cv. Annogiri) to varying rates (0, 200, 500, and 800 ppm) of lead [used as $Pb(NO_3)_2$] in order to quantify the damage expressed as the rate of lipid peroxidation, antioxidative responses, and the accumulation of lead in roots and shoots of both plants. A concentration-dependent increase in the measured parameters was observed. Plants grown with lead displayed increased levels of lipid peroxidation which was expressed in the form of enhanced malondialdehyde contents in roots and leaves of both plants along with the increase in the activities of SOD, CAT, POD, GR, and glutathione S-transferase (GST) compared to untreated plants. Of the different concentration tested, 800 ppm of lead had maximum activity and increased SOD, CAT, and POD by two- to three fold, GR activity by three- to five fold, and GST activity by three- to four fold in roots and leaves of both tested plants. While comparing the effect of lead, horse gram accumulated lead poorly compared to Bengal gram, but the contents of lead were greater in roots than leaves of both

plants. Similarly, copper treatment increased the activity of SOD, POD, and CAT in leaf tissue of bean (cv. Akman) as reported by Yurekli and Porgali (2006). Likewise, Metwally et al. (2005) observed that the concentrations of nonprotein thiols (NPTs), MDA, activity of chitinase, POD, and CAT significantly increased in pea genotypes treated with cadmium. Ascorbate peroxidase (APX) activities were however reduced, but the concentrations of GSH increased in the less cadmium-sensitive pea genotypes. Cadmium application also inhibited the uptake of nutrient elements, such as P, K, S, Ca, Zn, Mn, and B, by plants in an organ and genotype-specific manner. Wani et al. (2008a) also observed reduction in the GR activity in pea plants grown in the presence of nickel and zinc but in the absence of bioinoculant, while copper interfered with oxidative enzymes of bean leaves (Shainberg et al. 2001). All these enzymes play crucial role in physiological events of plants, and therefore, any change in their concentration within cells could lead to altered biochemical reaction. For example, variation in peroxidase activity following stressor effects has been reported to cause changes in respiration, photosynthesis, transpiration, and gaseous exchange events of plants (MacFarlane and Burchett 2001). Also, soybean growth with excess Fe resulted in increased O_2^- and $HO^{\bullet}$ production (Caro and Puntarulo 1996). Autooxidation and Fenton reaction may cause the oxidative loss of defense enzymes. For example, catalase activity is directly inhibited by O_2^- (Kono and Fridovich 1982). Cu–Zn superoxide dismutase is fragmented by hydroxy-radicals (Casano et al. 1997). If uptake of excess Fe^{2+} or Cu^+ preferentially derives the formation of hydroxy-radicals, protection mediated by antioxidant enzymes is unlikely (Polle 1997). Induction and activation of SOD and of antioxidant CAT are some of the major metal detoxification mechanisms in plants (Shanker et al. 2003a). For example, Molina et al. (2008) in a study assessed the cadmium-induced oxidative stress and antioxidant defense mechanisms in different organs of *Vigna mungo* L. For this, seeds were germinated in perlite–vermiculite using Hoagland nutrient solution. Six days after growth, seedlings were treated with 40 µM cadmium under semihydroponic conditions for 12 days. The antioxidative defense and oxidative parameters measured for roots, stems, and leaves were variable and tissue specific. Superoxide dismutase and guaiacol peroxidase (GPx) activities decreased in roots, but they increased in leaves. Catalase activity was also depressed following cadmium application. Total glutathione, nonprotein thiols, reduced glutathione (GSH), and phytochelatins were enhanced significantly, while oxidized glutathione (GSSG) declined relative to control plants. This finding therefore suggested that cadmium when present in soil or water can cause oxidative damage which may be harmful for mung production in contaminated environment. In contrast, Gwozdz et al. (1997) found that at lower heavy metal concentrations, activity of antioxidant enzymes increased, whereas at higher concentrations, the SOD activity did not increase further while CAT activity decreased. Pea plants exposed to environmentally relevant (20 µM) and acute (200 µM) concentrations of Cr(VI) for 7 days affected total SOD activity of root mitochondria differently. At 20 µM Cr(VI), SOD activity was found to increase by 29%, whereas 200 µM Cr(VI) produced a significant inhibition (Dixit et al. 2002). A decline in the specific CAT activity with increase in chromium concentration

from 20 to 80 ppm was observed (Jain et al. 2000). Samantaray et al. (1999) used POD and CAT activities as enzyme markers for identifying Cr-tolerant mung bean cultivars.

Conclusion

Although some heavy metals are required for physiological functions of living organisms, the excessive accumulation of such metals in various organs is always detrimental. Heavy metals cause toxicity to plants including legumes at different stages of growth and development. From various published data, it is plainly apparent that heavy metals exhibit toxicity at varying concentrations and that the lethal effect is plant genotype dependent. Generally, the noxious metals disrupt membrane functions and permeability, inactivate proteins and enzymes, and harmfully affect photosynthetic events and other metabolic process, damage cells by acting as antimetabolites, or form precipitates or chelates with essential metabolites. Further research is however required to better understand the toxicity of heavy metals to different physiological functions at molecular level.

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Chromium–Plant-Growth-Promoting Rhizobacteria Interactions: Toxicity and Management

4

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Abstract

Among heavy metals, chromium is a highly toxic nonessential metal found in different environmental settings. Chromium pollution has been reported worldwide, causes undeniable damage to microbes and plant genotypes, and is carcinogenic and genotoxic for humans. Of the two most common oxidative states, hexavalent chromium is relatively more deleterious than the less-mobile trivalent form of chromium. Chromium toxicity, however, can be reduced by employing various physicochemical and biological processes. Among biomaterials, apart from plants, use of plant-growth-promoting rhizobacteria has been found effective, inexpensive, and environmentally friendly. Plant-growth-promoting rhizobacteria alleviate the metal toxicity by adopting different strategies like biosorption and bioaccumulation, bioreduction to a less-toxic state, and chromate efflux. Some of these methods have been proposed as effective biological tools for removing chromium from contaminated locations. The interaction of chromium with plant-growth-promoting rhizobacteria and the bacterial-based management of chromium toxicity is reviewed and discussed. The detoxification of chromium by plant-growth-promoting rhizobacteria is likely to reduce the adversity of chromium to various agroecosystems and may serve as a good candidate for bacterial-based bioremediation of chromium-polluted soils.

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4.1   Introduction

Recently, the soil contamination by chromium has become one of the major concerns for scientists around the world. Globally, 10^7 tons of chromium is produced every year and about 60–70% of it is used in alloys, including stainless steel, and 15% in chemical industrial processes, mainly leather tanning, pigments, electroplating, and corrosion protection (Cheung and Gu 2007). Due to lack of proper disposal facilities, the environment becomes hugely contaminated by chromium (Sarangi and Krishnan 2008). The concentration of chromium in soils, for example, may vary between 5 and 3,000 μg of chromium per gram. In natural environment, chromium exists in multiple valence states, of which the trivalent and hexavalent chromium are the most common forms. Trivalent chromium is universally found in the environment and occurs naturally, whereas hexavalent chromium is released into the environment mainly through human activities. The resulting higher level of chromium has led to the destruction of agricultural lands and water bodies (Armienta-Hernändez and Rodriguez-Castillo 1995; Khasim et al. 1989) causing indirectly fatal and long-term toxic effects on humans and directly/indirectly on soil fertility (Viti 2006). In addition, the hexavalent chromium is mutagenic and carcinogenic (Desai et al. 2008; Costa and Klein 2006; Costa 2003) while trivalent chromium does not migrate freely in natural systems, because it readily precipitates as Cr(III) minerals or is removed by adsorption (Richard and Bourg 1991). Therefore, this is imperative to reduce the metal toxicity, which can be achieved by (a) identifying the place of pollutant's origin and treating pollutant at sites, if possible, (b) avoiding crop cultivation under such conditions so that the direct (through foods ingestion) or indirect (through feeds like fish and animals) transfer of metal toxicity could be checked, and (c) carefully and consistently monitoring and detecting polluted sites. Therefore, to protect soil and preserve natural integrity of soil ecosystems from toxic effects of pollutants including chromium, various physiochemical processes have been applied to remove/destruct heavy metals from the contaminated areas. These methods have been found poorly effective and prohibitively expensive, generate toxic secondary wastes that require further remediation, and become even ineffective for low-metal-contaminated locations (Wang and Chen 2009; Gavrilescu 2004). In addition, these methods are generally not metal specific. Biological approaches quite often referred to as bioremediation in contrast offer advantages like they can be used to remove metals selectively and provide operational flexibility. Furthermore, biological processes can be used both in situ or ex situ and are easy to operate, less expensive, do not produce secondary pollutants, and can effectively be applied even at poorly metal-polluted sites (De et al. 2008; Chen et al. 2005). Among various bioremediation strategies (Singh and Prasad 2011), a few utilize microorganisms which play pivotal role in the biogeochemical cycling of elements in soils and can transform toxic metals and radionuclides to less-disruptive forms (Das and Mishra 2010; Khan et al. 2009; Thacker et al. 2006; Chen et al. 2005; Camargo et al. 2003). Rhizosphere microbes especially plant-growth-promoting rhizobacteria (PGPR) among heterogenous microbial communities reduce the metal toxicity by biosorbing

metals (biosorption), mobilizing metals through the excretion of organic acids or methylation reactions (bioleaching), immobilization or biomineralization, intracellular accumulation, and enzyme-catalyzed transformation (Abdel-Sabour 2007; Lloyd 2002). Interaction of heavy metals especially chromium with PGPR and the recent advances in how PGPR could detoxify the toxicity of metals are discussed.

4.2 Source of Chromium

Chromium, a highly toxic metal, is found in all phases of the environment including soil, water, and air. The concentration of chromium in soils is however generally low. The content and distribution of chromium depend largely on the types of soils in different agroecosystems. The level of chromium in soils could however be increased because of natural processes and human activities, such as fossil fuel combustion, mining, smelting, sludge amendment to soil, fertilizer application, and agricultural practices (Gilmour and Riedel 2009; Landa 2005). Industrial processes such as electroplating, pigmentation, catalyst for corrosion inhibitors, glass and canning, leather, etc., also add metals including chromium to soils (Singanan et al. 2007; Shiny et al. 2004; Barnhart 1997; Baldi et al. 1990). Therefore, the excessive chromium concentration in the environment may result from (1) chrome plating and polishing operation, (2) inorganic chemical production, (3) cooling tower and steel mill effluents, (4) wood processing facilities, (5) petroleum refineries, and (6) the tanning industries. Once added to soils, chromium persists in the environment probably because of greater input from various sources compared to losses from soils. This problem is likely to continue in the future, and so, soil pollution with elements like chromium is genuinely an ever-increasing problem. Due to solubility and its high availability to plants, chromium plays an important role in the various soil processes (Oewietlik and Trojanowska 2004; Kolembkiewicz 1999).

4.3 Chromium–PGPR Interactions

Microorganisms in general are one of the key components of soil ecosystems which play critical roles in determining the nutrient pool of soil and therefore greatly affect the fertility of soils. Among various microbial communities, PGPR in particular has been found extremely important that modifies the nutrient balance of various agroecosystems by taking part in the (1) cycling of elements in soil, (2) generating plant-growth-regulating substances, (3) maintenance of soil structure, (4) detoxification of toxic chemicals, and (5) the management of plant insect pests (Ahemad and Khan 2011). However, whenever there is any disturbance in soil either due to natural process or through human-induced activities, like discharge of metal from various sources or use of agrochemicals, microbial populations inhabiting the rhizosphere are the ones that are affected first. As a result, the fertility of soil and concomitantly the production of crops under such stressed soil systems are severely affected. Therefore, the studies directed toward understanding the

impact of metals especially on PGPR from agronomic point of view have received greater attention.

Due to widespread use in different industrial operations and its toxic properties, chromium has become one of the serious environmental pollutants among various metals. Chromium, a ubiquitous biosphere contaminant, has been reported extremely detrimental to microbes and their associated activities (Chatterjee et al. 2009; Megharaj et al. 2003; Shi et al. 2002). Of the various forms of chromium, hexavalent chromium, being soluble in water, moves rapidly in the subsurface and readily enters a cell, whereas the reduced form, trivalent chromium, is relatively insoluble and immobile and thus is not bioavailable and less toxic. Hexavalent chromium, for example, is taken up by the cells of sulfate-utilizing organisms through the membrane sulfate transport channels, and after entering inside the cell, Cr(VI) can oxidatively damage biomolecules like DNA and other cellular components (Kamaludeen et al. 2003). By producing more reactive intermediate species like Cr(V) and Cr(IV), which through their toxic properties, chromium (VI) can cause mutagenic and carcinogenic effects on biological systems. The elevated concentration of heavy metals can inhibit enzymatic activities by (1) interacting with the enzyme substrate complexes, (2) denaturing the enzyme protein, and (3) interacting with its active sites (Shun-hong et al. 2009). Metals also affect soil enzymatic activities indirectly by changing the heterogenous microbial communities inhabiting the rhizosphere, capable of synthesizing such enzymes (Belyaeva et al. 2005). The toxicity of chromium to various PGPR like *Bacillus* spp. (Ibrahim et al. 2011; Dhal et al. 2010; Wani et al. 2008), *Pseudomonas aeruginosa* (Kiliç et al. 2010), asymbiotic bacteria like *Azotobacter* (Wani et al. 2008), and symbiotic organisms like *Rhizobium* (Wani et al. 2008, 2009; Joseph et al. 2007) are reported. In a study, Shun-hong et al. (2009) observed that the elevated chromium concentrations altered the activity of the soil microbes which was indicated by a negative correlation found between soil microbial populations and chromium contents. Among microbial activities, dehydrogenase activity was greatly depressed by chromium in the soil. It was therefore suggested from this study that the reduction in dehydrogenase activity in chromium-polluted soils could serve as an indicator for the chromium pollution as determined in the area of chromium-containing slag heap of steel alloy factory in China. Chromium toxicity to soil dehydrogenases, urease, and acid and alkaline phosphatases and the number of *Azotobacter* sp. is also reported (Wyszkowska et al. 2001). In other study, when *P. aeruginosa* was grown with varying concentration of chromium, the bacterial growth was decreased with increasing concentrations of Cr(VI) and a lag period of bacterial growth was observed at concentration higher than 20 mg l^{-1}. In contrast, Cr(VI) at 10 mg l^{-1} had a poor effect on bacterial growth, but beyond 40 mg Cr (VI) l^{-1}, only a negligible growth was recorded. The effect of Cr(VI) on the cell morphology of *P. aeruginosa* was examined further using SEM by growing this strain in liquid medium treated with or without Cr(VI) for 36 h. The morphologies of cells did not remain intact, the cell size increased in the presence of Cr(VI), and the binary cell fission was observed when this bacterium was exposed to 40 mg l^{-1} Cr(VI) (Wei-hua et al. 2009). Therefore, based on these and other reports, the

toxicity of chromium to various PGPR must be checked under in vitro conditions before they are applied as inoculants for raising the crop under chromium-stressed environments. Moreover, if soils are contaminated with metals, they can be made suitable for cultivation by employing various remediation technologies. Some of these technologies are briefly discussed in the following section.

4.4 Bioremediation: A General View

Making soil free from contaminants and to restore polluted sites suitable for crop production by using biomaterials is indeed an exciting and inexpensive process. In order to achieve such objective, bioremediation has been employed in situ (involves the treatment of pollutants at the site of origin) and ex situ (involves the treatment of contaminated soil that is collected from a poisoned site). These methods in general have resulted in considerable success due in part to the public acceptance and support, success rates, and comparatively low cost. However, like any other technology, bioremediation also has certain disadvantages, such as unpredictable success due to complex and variable biological system, and bioremediation very rarely restores the degraded land to its original state. For example, the residual contamination left after treatment is strongly sorbed on to soil constituents, and hence, they may become uninhabitable for some microbes and therefore are not available for degradation by soil microbial communities. In addition, the residues accumulated in soils over the years may lead to additional pollution. Despite all these constraints, bioremediation is considered a viable option for alleviating the metal toxicity from contaminated environment. Probably, the better efficiency and the low cost are the factors that make the biological approaches a very exciting option relative to conventional physicochemical methods for heavy metal removal from contaminated sites (Dary et al. 2010; He et al. 2010; Braud et al. 2009; Vaxevanidou et al. 2008). Bioremediation as a technique may involve both plants (generally termed phytoremediation) and microbial communities (Antizar-Ladislao 2010; Khan et al. 2009) for heavy metal decontamination from polluted soils. However, only the role of biomaterials especially PGPR in the management of metal especially chromium-contaminated sites is highlighted in the following section.

When applied, microorganisms including PGPR reduces the availability and toxicity of heavy metals in soils (Karami and Shamsuddin 2010; Khan 2005). Since rhizosphere due to high concentration of nutrients exuding from the roots supports the growth of microbes, the metabolically active microbes in turn facilitate the growth of the plants by affecting biogeochemical cycling of soil constituents and by other mechanisms, discussed earlier (Abbas-Zadeh et al. 2010; Wenzel 2008). Therefore, the plant–bacterial system has been found more effective in alleviating the toxicity of metals from contaminated soils. In this context, different processes such as (1) biostimulation—stimulation of viable native microbial population, (2) bioaugmentation—artificial introduction of viable population, (3) metal reduction, (4) biotransformation, (5) bioaccumulation—use of living cells, (6) biosorption, and (7) use of dead microbial biomass have been tested in bioremediation

technologies. Each of these methods plays important roles in decontaminating metal-polluted environment and can remove metal selectively with operational flexibility (De et al. 2008; Hallberg and Johnson 2005). Of these different bioremediation strategies, biosorption, for example, when applied properly can reduce capital, operational, and total treatment costs by 20, 36, and 28%, respectively, relative to conventional processes (Loukidou et al. 2004). A few examples of metal-removing/reducing ability of certain PGPR are listed in Table 4.1.

4.5   Management of Chromium Toxicity Using PGPR

Since hexavalent chromium has shown high degree of toxicity to various organisms including bacterial population especially PGPR, reducing Cr(VI) to Cr(III) has therefore become extremely important for the safety of the environment. In order to survive under such contaminated environment, bacterial species have, however, evolved certain mechanisms. As an example, *Ochrobacterium tritici* strain 5bvl1, a highly Cr(VI)-resistant model bacterium, is reported to contain the transposon-located (Tn*OtChr*) chromate resistance genes such as *chrB*, *chrA*, *chrC*, and *chrF*. Of these, only *chrB* and *chrA* genes are required essentially for the establishment of high resistance in this bacterium. However, other mechanisms involved in chromium resistance in this strain were largely associated with their ability to reduce Cr(VI), free-radical detoxifying activities, and ability to repair damaged DNA.

Table 4.1 Some examples of PGPR involved in bioreduction of heavy metals

Bacteria	Heavy metals	Role of PGPR	References
Bacillus sp.	Cr(VI)	Reduced Cr(VI) to Cr(III)	Cheng and Li (2009)
Cellulosimicrobium cellulans	Cr(VI)	Reduced chromium under aerobic culture condition and reduced the uptake by chilly plants	Chatterjee et al. (2009)
Bradyrhizobium japonicum	As	Reduced As, stimulated growth of soybean, and decreased arsenic absorption	Reichman (2007)
Ochrobacterium intermedium	Cr(VI)	Increased plant growth and decreased Cr(VI) uptake	Faisal and Hasnain (2005)
Pseudomonas fluorescens	Hg	Reduced Hg, increased plant growth	Gupta et al. (2005)
Ochrobacterium, Bacillus cereus	Cr(VI)	Lowers the toxicity of Cr to seedlings by reducing Cr(VI) to Cr(III)	Faisal and Hasnain (2006)
Mesorhizobium	Cr(VI)	Reduced Cr(VI) and decreased the concentration of Cr(VI) in plant parts	Wani et al. (2008)
Bacillus sp.	Cr(VI)	Reduced Cr(VI) to less-toxic form	Wani et al. (2007a)
Mesorhizobium sp.	Cr(VI)	Reduced Cr(VI)	Wani et al. (2009)

Expression of the *chrB*, *chrC*, or *chrF* genes was related to increased resistance to superoxide-generating agents. Further genetic analyses revealed that the *ruvB* gene is related to chromium resistance in *O. tritici* 5bvl1. When *ruvB* gene is interrupted, the RuvABC complex is not formed, and hence, the repair of DNA damage induced by chromium is prevented (Morais et al. 2011). However, in many cases, since Cr cleanup programs are based largely on the availability of Cr(VI) in the soils, most of the available treatment technologies emphasize on (1) removing directly the Cr (VI)-polluted soils, (2) immobilizing the chromium to avoid leaching after treatment under natural field conditions, and (3) transforming/reducing the toxic state of chromium (Cr VI) to stable, less soluble, and less-toxic Cr(III) state in stressed soils. Of these, reduction of toxic Cr(VI) to Cr(III) is considered an effective and more attractive option for restoration of Cr(VI)-affected environments (Jeyasingh and Philip 2005). To this end, certain conventional methods like chemical oxidation or reduction, ion exchange or adsorption, chemical precipitation, filtration, electrochemical treatment, reverse osmosis, evaporation recovery, and membrane technologies (Ahluwalia and Goyal 2007; Zahoor and Rehman 2009) have widely been applied, but these methods have been found both expensive and disruptive. Considering the deleterious impact of certain physicochemical methods and need to identify alternative technologies for reducing/destroying chromium toxicity, the researchers in recent times have directed their focus on abatement of Cr(VI) toxicity by using PGPR (He et al. 2011; Chaturvedi 2011; Wei-hua et al. 2009; Ozturk et al. 2009; Cheng and Li 2009; Li et al. 2007). In contrast to the conventional methods, the use of microbes in remediation technologies is easy, less costly, and environmentally safe and provides a viable and sustainable option to protect the environment from chromium hazards. The bioremediation system in operation today therefore stresses greatly on the use of microorganisms which could be recovered either from conventional soils or soils contaminated with chromium. Regardless of whether the PGPR are indigenous or introduced from outside to the contaminated locations, an understanding of how they remove/destruct contaminants is critical to understanding the various bioremediation strategies. This aspect is briefly discussed in the following section.

Microorganisms inhabiting soils and rhizosphere are known to play significant roles in the bioremediation of heavy metal including chromium-contaminated soil (Khan et al. 2009; Faisal and Hasnain 2005). Like any other indigenous soil microbes, PGPR are also well equipped to survive in chromium-contaminated locations and also possess the ability to significantly reduce the toxicity of Cr(VI) in highly polluted soil which may sometimes be uninhabitable for other microbes. For example, some of the microbial communities including *Brucella* sp. (Urvashi et al. 2007), *Leucobacter* sp. CRB1 (Zhu et al. 2008b), and *Bacillus* sp. (Mary Mangaiyarkarasi et al. 2011), isolated from different agroclimatic regions, have shown tolerance to chromium to a level of around 100–4,000 mg l^{-1} Cr(VI). However, the variation in tolerance to heavy metals has been attributed to varying chemical composition of the medium used, which probably help to mask the entry of toxicants inside bacterial cells (Desai et al. 2008; Caravelli et al. 2008). However, when grown in liquid culture containing heavy metals, bacterial cells in

general are more sensitive to metal toxicity due in part to the excess and free availability of metals in liquid than in solid medium (Shakoori et al. 2000). When such chromium-tolerant microbes are applied intentionally in contaminated soils, hexavalent chromium is reduced to a very stable chromium form (trivalent chromium), with a minimal risk of rerelease of Cr(VI) into the environment. Of the various bacterial cell components, cell walls and membranes, which are able to prevent hexavalent chromium out from living cells, have been found as the main sites where hexavalent chromium reduction occurs, as observed in case of *Bacillus cereus* S5.4, a Gram-positive bacterium isolated from the electroplating sludge of Baosteel Corporation, Shanghai. Further, changes in the permeability of cell wall or membrane are likely to affect the function of hexavalent chromium reductase (Xiao et al. 2008). Among variously distributed microbial genera, PGPR like *Bacillus*, *Pseudomonas*, *Azotobacter*, and rhizobia are reported to restore chromium-contaminated sites (Karami and Shamsuddin 2010; Wani et al. 2007a, 2008). In this context, heavy-metal-resistant bacteria isolated from the soil samples of tanning industry were used to assess the hexavalent chromium bioaccumulation ability and subsequently to evaluate their ability to remove Cr(VI) from tannery effluents (Seng and Bielefeldt 2002). The chromium reduction was significantly influenced by the pH of the effluent and was attributed to the cellular growth of the bacteria *Pseudomonas*. The reduction of chromium (VI) by the bacterial species was enzyme-mediated which resulted in the formation of reactive intermediates and Cr(III) (Seng and Bielefeldt 2002). In a similar study, Rehman et al. (2008) reported the biotransformation of hexavalent chromium into its trivalent form by *Bacillus* species. Interestingly, these bacteria could reduce 91% of chromium added to liquid medium 96 h after growth and reduced 84% chromium found in the industrial effluents collected from the province of Lahore, Pakistan, after 144 h (Rehman et al. 2008). Likewise, Dhal et al. (2010) isolated a bacterial culture identified as *Bacillus* sp. based on standard biochemical tests and partial 16S rRNA gene sequencing, which was tolerant to 2,000 mg l^{-1} Cr(VI). This strain was further found to reduce Cr(VI) to Cr(III), when grown in media treated with hexavalent chromium. At the optimum conditions like pH 7, 100 mg l^{-1} Cr(VI), 35°C temperature, and shaking speed 100 rpm, strain CSB-4 reduced more than 90% of Cr(VI) in 144 h. The time course reduction data fitted well to an exponential rate equation yielding rate constants in the range 3.22×10^{-2} to 6.5×10^{-3} h^{-1} for Cr(VI) concentration of 10–500 mg l^{-1}. The activation energy derived from temperature dependence rate constants between 25 and 35°C was found to be 99 kJ mol^{-1}. Since the discovery of the first bacteria (*Pseudomonas dechromaticans*) capable of reducing Cr(VI) in the 1970s (Romanenko and Korenkov 1977), numerous bacterial genera such as *Pseudomonas* spp. (Jimenez-Mejia et al. 2006; Ganguli and Tripathi 2002; Mclean and Beveridge 2001; Rajwade et al. 1999; Wang and Xiao 1995), *Brevundimonas* spp. (Lu et al. 2011), *Shewanella* sp. (Guh et al. 2001; Myers et al. 2000), *Achromobacter* spp. (Zhu et al. 2008a; Wani et al. 2007b; Ma et al. 2007), *Bacillus* spp. (Ibrahim et al. 2011; Mary Mangaiyarkarasi et al. 2011; Zahoor and Rehman 2009; Okeke et al. 2008; Elangovan et al. 2006; Wang and Xiao 1995), *Vogococcus* sp. (Mistry et al. 2010), and other bacterial genera (Sultan and Hasnain 2007; Thacker

et al. 2006; Puzon et al. 2005; Pal and Paul 2004) capable of reducing chromium under both aerobic and anaerobic environment have been identified and tested. A few examples of PGPR involved in chromium reduction and their consequent impact on various crops are listed in Table 4.2. Based on these and other unreported

Table 4.2 Plant-growth-promoting rhizobacteria affecting remediation of chromium

Mechanism	Organism	Description and effectiveness	References
Bioreduction	*Pseudomonas putida* P18 and *P. aeruginosa* P16	Reduction by *Pseudomonas* strains was due to the release of constitutive reductases that intracellularly and/or extracellularly catalyzed the reduction of Cr(VI) to Cr(III)	Dogan et al. (2011)
Bioreduction	*Pseudomonas corrugata* 28	Reduction of Cr(VI) by *P. corrugata* 28 mainly led to the formation of dissolved organic Cr(III) complexes	Christl et al. (2011)
Bioreduction	*Bacillus* sp.PSB10	Reduced the uptake of chromium in roots, shoots, and grains of chickpea; significantly improved growth, nodulation, chlorophyll, leghemoglobin, seed yield, and grain protein of chickpea grown in the presence of different concentrations of chromium	Wani and Khan (2010)
Bioreduction	*P. aeruginosa*	Cr(VI) was reduced from 40 mg l^{-1} to about 18 mg l^{-1} in 72 h. The value of pH dropped from 7.02 to around 5.65 after 72 h. A significant increase in the value of redox potential occurred during Cr(VI) reduction, and Cr(VI) reduction was observed over a range of redox potential from +3 mV to +91 mV. Both SO_4^{2-} and NO_3^- had no effect on Cr(VI) reduction	Wei-hua et al. (2009)
Bioreduction	*Pseudomonas* sp.	Maximum reduction of 1,125 ppm was recorded with a 305 V/V inoculum concentration	Rahman et al. (2007)
Bioreduction	*Bacillus* spp.	Maximum chromate reduction (87%) was achieved at pH 7 at 37°C after 120 h incubation and initial concentration of Cr(VI) at 100 μg ml^{-1}. A 50 μg Cr(VI) ml^{-1} was completely reduced after 100 h	Wani et al. (2007a)
Bioreduction	*Ochrobacterium intermedium*	Reduced 68% Cr(VI) to Cr(III) in nutrient solution after 10 days at an initial concentration of 300 μg K_2CrO_4	Faisal and Hasnain (2005)
Bioreduction	*Bacillus* sp.	Anaerobically reduced 90% of Cr(VI) in 6 h	Camargo et al. (2003)
Bioreduction	*Pseudomonas*	Completely reduced 20 μg ml^{-1} chromate after 120 h	McLean and Beveridge (2001)
Bioreduction	*Microbacterium liquefaciens*	Removed 100 μM Cr(VI) within 96 h	Pattanapipitpaisal et al. (2001a, b)

data (in this chapter) but surveyed, it is suggested that chromium-resistant microorganisms or organisms able to reduce chromium toxicity can serve as a promising candidate for detoxification of sites contaminated heavily with metals including chromium (Morales et al. 2007). Even though bacteria able to detoxify metals has been tested in different systems on numerous occasions, the survivability and physiological functions of cells under metal-stressed environment have still been a challenge which needs immediate attention so that the efficiency of microbes and consequently the bioremediation potentials of PGPR can be improved (Cheung and Gu 2007).

4.6 Mechanism of Hexavalent Chromium Reduction

4.6.1 Direct Mechanism

Microbial communities are known to reduce the chromium toxicity both directly, for example, by *Pseudomonas* sp. (Desai et al. 2008) and indirectly by *Thiobacillus thiooxidans* (Donati et al. 2003).The mechanism of reduction of hexavalent chromium by microorganisms and their bioremediation potential has recently been reviewed by Cheung and Gu (2007). In the direct mode, the microbial communities in general take up chromium while growing in the chromium-contaminated sites and then reduce it enzymatically by chromium reductases synthesized by them (Chirayu et al. 2008; Pal et al. 2005). Chromium reductase catalyzes the transformation of Cr(VI) to Cr(III) anaerobically (Zhu et al. 2008a, b; Lovley and Phillips 1994), aerobically (Zemin et al. 2007; Pal and Paul 2004; Cerventes et al. 2001), and sometimes also both anaerobically and aerobically (Marsh and Mclnerney 2001). The Cr(III) so generated by diverse bacterial species may further be detoxified by other mechanisms (Ramírez-Díaz et al. 2008). The enzyme reductase affecting chromium transformation has been reported in the cell membrane fractions of PGPR like *Pseudomonas fluorescens* and *Enterobacter cloacae* (Wang et al. 1990) or soluble fraction of bacterial cells, for example, in *Bacillus sphaericus* AND 303, isolated from serpentine soil of Andaman, India (Pal et al. 2005). In a follow-up experiment, Ilias et al. (2011) noticed a similar chromate reductase activity in the culture supernatant and cell lysate of *Staphylococcus aureus* (IFR-2) and *Pediococcus pentosaceus* (IFR-3). Whole cells of IFR-2 and IFR-3 reduced 24% and 30% of the initial Cr(VI) concentration (1 mg l^{-1}) in 45 min, respectively, at 37°C. The optimum temperature and pH for growth of bacteria and Cr(VI) reduction by both isolates ranged between 35°C and 40°C and pH 7–8. In addition, chromium reductase activity has also been reported in cytoplasmic membrane of bacteria as found in anaerobically grown *Shewanella putrefaciens* MR-1 (Park et al. 2000). These chromium reductases have been purified and characterized as in the case of *Bacillus* sp. (Wang et al. 1990) or

P. putida (Puzon et al. 2002). The reductase activity was NADH- or NADPH-dependent. However, most characterized enzymes belong to the widespread NAD (P)H-dependent flavoprotein family of reductases. The other mechanism that is adopted by bacterial cells to overcome the chromium toxicity while growing in undesirably higher concentration of chromium is chromate efflux by which chromate ions are pumped out from the cell cytoplasm and hexavalent chromium is reduced to Cr(III). For example, chromate efflux by the ChrA transporter has been established in *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) and consists of an energy-dependent process driven by the membrane potential (Ramírez-Díaz et al. 2008). In other experiment, Cr(VI)-reducing bacterial strain MCMB-821 later identified as *Burkholderia cepacia* was isolated from the alkaline crater lake of Lonar. This strain when grown with 2% salt and lactose (as electron donor) and variable concentrations of chromium survived well (resistance) up to 1,000 ppm Cr(VI) and reduced 98% of the 75 ppm Cr(VI) within 36 h at pH 9. The chromate-reducing efficiency of MCMB-821 was comparable under both aerobic and anaerobic conditions. Further analysis by electron paramagnetic resonance spectroscopy revealed that this strain was able to reduce Cr (VI) to Cr(III) via the formation of transient Cr(V) intermediate. However, membrane inhibitors negatively affected the chromate-reducing ability of strain MCMB-821, but reducing potential was enhanced when 2,4-dinitrophenol was applied. This finding therefore suggested that the electron transport chain also plays an important role in the bacteria-based reduction of Cr(VI), as reported by Wani et al. (2007b). While comparing the effect of liquid and solid media on bacterial growth and to determine differences in chromium-reducing ability of microbial population, Pei et al. (2009) conducted an experiment using actively growing cells of *Acinetobacter haemolyticus*. From this experiment, it was observed that the bacterium *A. haemolyticus* grew comparatively better in liquid broth than solid agar medium as was evident from the fact that the strain tolerated 90 mg Cr(VI) l^{-1} in LB broth compared to only 30 mg Cr(VI) l^{-1} in LB agar. The FTIR analysis further showed that the Cr(III) species formed was also most likely to form complexes with carboxyl, hydroxyl, and amide groups of the bacteria. However, no precipitates were noticed on the cell wall region of the bacteria by TEM, but microprecipitates were seen in the cytoplasmic region of the cells, suggesting the migration of Cr(VI) inside the cells. Later on, when cell-free extracts were used to detect intracellular chromium reduction ability of this strain, specific reductase activity obtained was 0.52 μg Cr(VI) reduced per mg of protein per hour at pH 7.2 and 37°C. This finding also validated the facts that bacterial species in general and *A. haemolyticus* in particular could be of practical value in reducing the chromium toxicity in the chromium-enriched locations. The hexavalent chromate reductase activity may also be found in, for example, cytosolic fraction of a bacterial cell like in the case of *Pseudomonas* sp. G1DM21 and may catalyze the bioreduction of hexavalent chromium (Desai et al. 2008). When tested under in vitro conditions, 99.7% of 500 μM Cr(VI) and 93% of 1,000 μM Cr(VI) were reduced by the suspended culture of the strain G1DM21 in 48 h, while it consistently reduced 100 μM Cr(VI)

within 6 h up to four consecutive inputs. Of the various cell preparations, the permeabilized cells of the bacterium could reduce 92% within 6 h while cell-free extracts (CFE) could reduce 90% of 100 μM Cr(VI) in 2 h. The K_m and V_{max} values of chromate reductase activity in the CFE were 175 μM Cr(VI) and 1.6 μmoles min^{-1} mg^{-1} of protein, respectively, the K_m and V_{max} determined in the presence of 0.5 mM NADH were 150 μM Cr(Vi) and 2 μmoles min^{-1} mg^{-1} of protein, respectively. Hexavalent chromate reductase activity was maximum at 30°C and pH 7. The relative molecular mass (M_r) of the native Cr(VI) reductase in the cytosolic fraction was estimated as 61.7 kDa. Further, the Cr(VI) reductase activity was enhanced in the presence of metal ions like Cu^{2+}, Mg^{2+}, and Na^+ and electron donors (like citrate, succinate, acetate) and was profoundly suppressed in the presence of metal ions like Hg^{2+}, Ag^+, and Cd^{2+} and disulfide reducers like 2-mercaptoethanol. The respiratory inhibitors in contrast exhibited a poor effect on the enzyme activity. When this bacterium was grown with 1 mM Cr(VI) for 24 h and observed under scanning probe atomic force microscopy (AFM), there was an increase in cell length and height of the tested bacterial cell (Desai et al. 2008). Other mechanisms of bacterial resistance to chromate involve the expression of components of the machinery for repair of DNA damage and systems related to the homeostasis of iron and sulfur (Ramírez-Díaz et al. 2008).

4.6.2 Indirect Mechanism of Chromium Reduction

While using indirect route, the reductants or oxidants, such as H_2S, which are released by microbial cells into soil, play an important role in the reduction of chromium toxicity by chemical redox reactions (De Filippi and Lupton 1992). For example, *Thiobacillus ferrooxidans* and *T. thiooxidans*, growing on elemental sulfur, were found to indirectly promote chromium (VI) reduction by the production of reducing agents such as sulfite and thiosulfate (Donati et al. 2003). Similarly, other species of *Thiobacillus*, like *T. thioparus*, were tested for their bioreduction ability while growing in a fermentation vessel containing medium supplemented with sulfur (as the sole energy source) at 30°C with shaking at 400 rpm at three different pH (6, 7, or 8). The culture was maintained with automatic addition of KOH. Interestingly, *T. thioparus* completely reduced the chromium (VI) toward the end of growth period at all the three pH tested. Therefore, the reduction of Cr(VI) by H_2S secreted by bacterial cells, followed by precipitation of the Cr(III) formed, is considered a pivotal mechanism in sulfate-rich soil location under anaerobic conditions (Losi et al. 1994). Hydrogen sulfide, produced in acid sulfate soil under reducing conditions, is easily precipitated as FeS in reduced soils (Eary and Rai 1991) and sediments. Fe(II) and H_2S, both microbially produced, are effective reductants of Cr(VI) under reduced conditions as is the FeS (Karnachuk 1995).

4.7 Factors Affecting Chromium Reduction

4.7.1 pH

pH is one of the most important factors that plays significant role in the overall growth of microbial communities in varied ecological niches, and hence, also affects the various metabolic activities including the ability of variously distributed microbes to remove/degrade the toxic chromium from hugely contaminated locations. Chromium reduction by microbes has been observed at neutral (Wani et al. 2007a; Liu et al. 2004), alkaline (Mangaiyarkarasi et al. 2011; Shakoori et al. 2000), and acidic pH (Silva et al. 2009) by different bacterial genera. However, there are conflicting reports on the chromium reduction by microbes when grown at varying pH values. For example, PSB 10, PSB1, and PSB7 strains of plant-growth-promoting rhizobacteria *Bacillus* spp. maximally reduced the hexavalent chromium by 87%, 83%, and 74% when grown at pH 7 (Wani et al. 2007a), whereas *Enterobacter cloacae* showed maximum chromium-reducing ability at pH 6.5–8.5. The chromium reduction was, however, strongly inhibited both at pH 5 and 9 (Wang et al. 1990).

Bioreduction of toxic chromium at pH higher than normally required for bacterial growth is considered important for certain bioremediation efforts probably because alkaline soil has also been found contaminated with chromium. In this context, Mary Mangaiyarkarasi et al. (2011) reported that detoxification of Cr(VI) under alkaline pH requires attention due to the alkaline nature of many effluents as well. Therefore, certain bacteria, for example, a Gram-positive *Bacillus subtilis*, able to grow in alkaliphilic environment were isolated from tannery-effluent-contaminated soil, and later on upon investigation displayed the ability to grow well at alkaline pH and also reduced Cr(VI) up to 100% at pH 9. The XPS and FT-IR spectra confirmed the reduction of Cr(VI) by bacteria into chromium (III) which was mediated by membrane-bound chromate reductase. In a similar manner, the influence of varying levels of pH on Cr(VI) reduction yield and growth of Cr(VI)-resistant alkaliphilic bacteria isolated from sediment and water samples collected from Wadi Natrun hypersaline soda lakes (located in northern Egypt), which was later on identified using 16S rRNA gene analysis, as *Bacillus* sp. KSUCr5, was investigated (Ibrahim et al. 2011). The strain KSUCr5 could reduce Cr(VI), when grown at pH ranging between 7 and 12 with an optimum growth and reduction yield at pH 10, as also observed for *Ochrobacterium* sp. CSCr-3 (He et al. 2009), indicating the alkaliphilic nature of *Bacillus* sp. strain KSUCr5 (Horikoshi 1999, 2011). However, the chromium reduction was significantly decreased at pH 12 and was completely lost at acidic pH like at pH 5–6. Since chromium reduction by microbes is mostly carried through chromium reductase, therefore any variation in pH is likely to have effect on the activity of chromium-reducing enzymes.

4.7.2 Chromium Concentration

Hexavalent chromate [used as potassium dichromate ($K_2Cr_2O_4$)] reduction by *Bacillus* sp. KSUCr5 using different chromium concentrations ranging from 10 to 300 mg l^{-1} was investigated by Ibrahim et al. (2011). This bacterium completely reduced Cr(VI) within 24 h when the initial Cr(VI) concentration was up to 40 mg l^{-1} with chromate reduction rate of 1.7 mg h^{-1}. While with increase in chromium concentration, there was also an increase in time required for chromium reduction. And therefore, when 60–100 mg l^{-1} was added to medium, complete (100%) reduction of Cr(VI) occurred within 48–72 h. However, a total of 78% of 150 mg l^{-1} and 44% of 200 mg Cr(VI) l^{-1} were reduced within 72 h along the formation of visible white precipitate of Cr(III) at the bottom of the culture bottle. In a similar study, *Microbacterium* sp. was also found to completely reduce 20 mg l^{-1} Cr(VI) within 72 h (Pattanapipitpaisal et al. 2001a, b) while pseudomonad strain CRB5 completely reduced 20 mg l^{-1} of chromate after 120 h of growth (McLean and Beveridge 2001). On the other hand, *Bacillus sphaericus* AND 303 failed to completely reduce even 10 mg l^{-1} Cr(VI) as reported by Pal and Paul (2004) whereas 50 mg l^{-1} were reduced to zero in 54 h by *Brucella* sp. (Thacker et al. 2007). Among other microbes, certain halophilic strains, for example, *Nesterenkonia* sp. strain MF2 possessing highest chromate tolerating ability (600 mM), completely reduced 117.6 mg l^{-1} hexavalent chromium within 72 h, but beyond this concentration, there was no complete reduction even after 120 h (Amoozegar et al. 2007). Mangaiyarkarasi et al. (2011) has reported that alkaliphilic *B. subtilis* reduced 50, 100, 150, and 200 mg l^{-1} Cr(VI) to near zero, 71%, 62%, and 27%, respectively, in 65 h and 144 h (100 mg l^{-1}), respectively. A highly Cr(VI) proficient bacterial strain such as *Lysinibacillus fusiformis* reduced 1 mM Cr(VI) within 12 h (He et al. 2011). In a study, Okeke (2008) recovered a chromium-resistant bacterium, *Exiguobacterium* sp., 99% closely related to *Exiguobacterium acetylicum* capable of removing Cr(VI) and found it to reduce chromium significantly at both high and low concentrations (1–200 µg ml^{-1}) within 12 h. The Michaelis–Menten K_m and V_{max} for Cr(VI) bioremoval were calculated to be 142 µg ml^{-1} and 13 µg ml^{-1} h^{-1}, respectively. However, the bacterial growth did not differ at 1–75 µg ml^{-1} Cr(VI) until 12 h incubation. At 8 mg l^{-1}, *Exiguobacterium* sp. GS1, however, rapidly removed Cr(VI) with over 50% bioremoval after 3 h and 91% bioremoval after 8 h growth. In further experiments, the *Exiguobacterium* sp. GS1 grew luxuriantly and significantly reduced Cr(VI) when medium was treated with 1–9% salt, suggesting a high salt-tolerating ability of this strain. In addition, this strain also reduced Cr(VI) substantially over a wide range of temperature (18–45°C) and pH (6–9). The T_{opt} and pH_{opt} were 35–40°C and 7–8, respectively. It was therefore suggested from this study that the multiple properties of *Exiguobacterium* sp. GS1 could be exploited in bioremediation programs aimed at the removal of toxic chromium in complex and diverse agroecological regions of the world. Thacker and Madamwar (2005) in other experiment isolated a hexavalent chromium-reducing bacterial culture (DM1) from the contaminated sites of chemical industries and determined its ability to reduce

hexavalent chromium to trivalent chromium, using both cell suspension and cell extract. Based on the biochemical analysis, DM1 was identified as *Ochrobacterium* sp. and tolerated chromium to a level of 300 ppm with optimum temperature and pH for chromium reduction as 35°C and 7, respectively. The permeabilized cells of this bacterium treated with toluene and Triton X-100 and cell free extract of this culture demonstrated that the hexavalent chromium reduction was associated mainly with the soluble fraction of the cell. The chromium-reducing activity was inducible. The finding that this bacterium had an induced protein of molecular weight around 30 kDa when grown in the presence of chromium and also in cells when grown in the absence of chromium stress indicated a possible role of this protein in chromium reduction.

4.7.3 Effect of Temperature

Among different environmental variables, temperature is one of the major factors that affects the microbial-based Cr(VI) reduction. To further validate this concept, chromium reduction by a Gram-positive bacterium *Bacillus* sp. KSUCr5 at temperatures ranging between 25 and 50°C was studied (Ibrahim et al. 2011). Chromate reduction increased consistently with increasing temperature up to 35°C, which at 40°C was about 56% of the reduction yield observed at the optimum temperature (35°C). Microbial growth and reduction of chromium was, however, decreased dramatically above 40°C. In agreement to this finding, the optimal temperature of Cr(VI) reduction by microbes in general lies in the range of 30–37°C (Cheung and Gu 2007). Maximum Cr(VI) reduction by a Gram-positive moderately halophilic chromate-reducing bacterial strain, isolated from effluents of tanneries, and identified as *Nesterenkonia* sp. strain MF2 by phenotypic characterization and 16S rRNA analysis *Nesterenkonia* sp. strain MF2 (Amoozegar et al. 2007) and *Ochrobacterium* sp. CSCr-3 (He et al. 2009), was found to be 35°C, whereas for *Bacillus* sp. (Wang and Xiao 1995) and *Pseudomonas* strain CRB5 (McLean et al. 2000), it was 30°C. In addition, a few thermophilic bacteria like *Thermus scotoductus* SA-01 have also been shown to reduce chromium by chromate reductase at an optimum temperature of 65°C (Opperman et al. 2008).

4.7.4 Glucose and NaCl Concentration

Chromium-reducing bacteria are known to utilize numerous organic compounds as electron donors during Cr(VI) reduction (He et al. 2009; Liu et al. 2004). For example, Ibrahim et al. (2011) evaluated the effect of glucose on bacterial growth and their chromium-reducing ability and observed that Cr(VI) reduction was enhanced markedly when glucose was added to the growth medium. A concentration-dependent increase in bacterial growth and Cr(VI) reduction were determined following glucose application; the maximum growth and bioreduction yield (66%) was observed at 1% and about 1.5% glucose concentrations. However, further

increase in glucose level did not result in corresponding increase in Cr(VI) reduction. Glucose by acting as an electron donor has also been shown to efficiently increase Cr(VI) reduction by *Bacillus* sp. (Liu et al. 2006; Pal et al. 2005) and a rod-shaped, Gram-negative, and motile bacterium *Ochrobacterium* sp. CSCr-3 (He et al. 2009). Besides glucose, other electron donors like formate, fructose, and carbonate have also been reported to increase Cr(VI) reduction (He et al. 2011; Myers et al. 2000). Similarly, the bacterial strain like *Bacillus* sp. KSUCr5, when grown with varying concentrations of NaCl (0–20%), has shown a considerable increase in bacterial growth and Cr(VI) removal (Ibrahim et al. 2011). The maximum growth and reduction yield (82%) was observed when medium had 0–1.5% NaCl, after which both bacterial growth and reduction level decreased substantially. At 4%, 10%, and 20% NaCl concentrations, strain KSUCr5 reduced Cr(VI) by 44%, 35%, and 24%, respectively, when medium was treated with 100 mg Cr(VI) l^{-1}. In contrast, the complete reduction of 0.2 mM Cr(VI) was achieved after 24 h by halophilic *Nesterenkonia* sp. strain MF2 only when the concentration of NaCl increased from 0.1 to 1 M (Amoozegar et al. 2007).

Conclusion

The environmental risk of chromium pollution is pronounced in soils due to improper and untreated discharge of various industrial by-products or application of agrochemicals. It is therefore imperative to understand the fatal impact of long-term contamination of chromium on the functioning of agronomically important soil microorganisms inhabiting various agroecosystems. Indeed, microbes especially plant-growth-promoting rhizobacteria either alone or when they establish a close relationship (symbiosis) with certain plants like legumes have shown greater promise in circumventing the toxicity of various metals including chromium in the environment. Bacterial-based remediation of chromium toxicity from the contaminated locations has been found practically feasible on the one hand and inexpensive on the other hand. However, there are certain challenges like how the chromium-reducing activity of one particular bacterium could be extended to those which otherwise do not possess this ability. Furthermore, could bacteria play any role in predicting and restricting the movement of chromium to legumes, if applied as inoculants, so that the toxicity of chromium to the plants could be avoided? In these directions, molecular tools of biology could probably play any roles to make bacterial-based bioremediation more efficient and applicable.

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The Influence of Glutathione on the Tolerance of *Rhizobium leguminosarum* to Cadmium

5

Corticeiro Sofia, Pereira Sofia, Lima Ana, and Figueira Etelvina

Abstract

Rhizobia play an important role in agriculture and crop production as they induce nitrogen-fixing nodules on the roots of leguminous plants. Due to the injudicious use of fertilizers and industrial and domestic sludges, the heavy-metal contamination of soils is becoming one of the most concerning environmental problems, which negatively affects the soil microbial communities and consequently the crop productivity. Among the nonessential metals, cadmium (Cd) poses a major threat due to its high mobility and bioavailability. Cadmium affects the survival and the ability of rhizobia to form nitrogen-fixing nodules. The identification of mechanisms that improve rhizobial tolerance to Cd, its persistence in soil, and its ability to improve nodulation efficiency of rhizobia in Cd-contaminated soils is an important issue that requires urgent attention for maintaining fertility of soils polluted with metals. Here we discuss the influence of glutathione (GSH) on Cd tolerance of *Rhizobium leguminosarum* and have tried to establish the chronology of Cd tolerance mechanism. To understand this, several strains were screened for their Cd tolerance, and the effect of bacterial pregrowth in the presence of extracellular GSH was determined. Cadmium and GSH levels were also monitored over 72 h. The importance of GSH in Cd tolerance was confirmed by the intracellular levels of this tripeptide: GSH intracellular levels remained unaffected in the sensitive strain, yet it increased significantly in the tolerant strain. Moreover, GSH synthesis was induced by intracellular Cd levels; the addition of extracellular GSH had a protective effect toward Cd, particularly in the sensitive strains. These results lead to a better understanding of the metal tolerance mechanisms in free-living bacteria and are likely to improve the *Rhizobium*-plant symbiosis in heavy-metal-contaminated soils.

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5.1   Heavy-Metal Soil Contamination

The transformations that man has made in the landscape have long been accepted as a hallmark of civilization. However, the fact remains that many agricultural and industrial practices have been adversely affecting the environment by introducing several toxic compounds, such as heavy metals (Trajanovska et al. 1997; Pazirandeh and Mauro 2000). In the last decades, an increase of heavy-metal contamination in water and soils has been considered one of the most current troublesome environmental problems (Alloway 1995a; Giller et al. 1998). These elements are ubiquitous and persistent pollutants that are introduced into the soil environment through anthropogenic activities, such as smelters, mining, power station industry, and the application of metal-containing pesticides, fertilizers, herbicides, and sludges (Giller et al. 1989; McGrath et al. 1995; Saxena et al. 1999; Robinson et al. 2001; Carrasco et al. 2005). Atmospheric deposition of industrial dust, mining operations, incineration processes, burning of fossil fuels (Alloway and Steinnes 1999), and military activities also contribute massively to increase the concentration of heavy metals in soils (Pazirandeh et al. 1998; Robinson et al. 2001). Agricultural soils often deficient in nutrients require the addition of fertilizers and sludges for growth and development of plants. According to Alloway (1995b), phosphatic fertilizers are widely regarded as the most ubiquitous source of Cd (<500 mg kg^{-1}) in soils. Sewage sludge application to land is also very common, resulting in the improvement of the physical and chemical characteristics of soils (Abd-Alla et al. 1999; Obbard 2001), since sludges add substantial amounts of organic matter and inorganic nutrients, such as N, P, Ca, and Mg to soils (Chander and Brookes 1993). However, there has been a growing concern over the use of sludges due to their heavy-metal content and other potential toxic compounds from both industrial and domestic sources (Purchase and Miles 2001; Horswell et al. 2003), thus contributing to increased soil contamination. Heavy metals become irreversibly immobilized in soil components, causing toxicity to microorganisms, plants, animals, and humans (McGrath and Lane 1989; Wani et al. 2007a, b). Among the nonessential metals, Cd poses a major threat due to its high mobility and bioavailability and hence has become one of the reasons why heavy-metal contamination is so important for numerous scientific investigations.

5.2   Nitrogen Fixation by Rhizobia

Rhizobia are ubiquitous Gram-negative soil bacteria that have a substantial scientific and agronomic significance due to their ability to establish nitrogen-fixing symbiosis with legumes. The resulting symbiotic relationship is of major importance to the maintenance of soil fertility (especially N pool) and to feed livestock and populations in many developing countries. Even though diatomic nitrogen (N_2) constitutes 81% of the earth atmosphere, this form of N is not available to plants in this chemical form. However, N_2 may become available through a symbiotic relationship between bacteria and leguminous plants (Atlas and Bartha 1997;

Figueira 2000; Abbas and Kamel 2004). Estimates are that rhizobial symbioses, with a number greater than 100 important agronomical legumes, contribute to nearly half the annual quantity of N fixed biologically entering soil ecosystems (Somasegaran and Hoben 1994; Zahran 1999). Hence, biological nitrogen fixation (BNF) is often used to improve infertile agricultural soils. In many developing countries, the effective management of N is considered an essential element for agricultural sustainability (Rehman and Nautiyal 2002). The six billion people on earth consume on an average of nearly 11 g of N per person per day (Fink et al. 1999). Plant sources satisfy up to 80% of dietary needs of people living in the tropics and subtropic regions. With the world population steadily increasing and expecting to reach 9.4 billion in 2050, world food production rates need to be increased by at least 50% (Murchie et al. 2009). In this way, unprecedented increases in crop production will be needed if the current levels of dietary proteins and caloric intake are to be maintained. For this reason, and considering the importance of legumes in animal and human foods, attention must be paid to understand the effects that environmental stresses exert on *Rhizobium* populations (Ibekwe et al. 1995). Understanding the events that affect the survival and proliferation of rhizobial populations in soil and rhizosphere that limit their ability to form effective symbiotic associations with legumes and, consequently, that decrease soil fertility and crop production (Hirsch et al. 1993; Chaudri et al. 1993) is of extreme practical importance.

5.3   The Influence of GSH on *Rhizobium leguminosarum* Tolerance to Cadmium

Mechanisms of Cd tolerance in bacteria are diverse and may involve energy-dependent efflux of ions (Purchase et al. 1997; Nies et al. 1998; Peitzsch et al. 1998; Goldberg et al. 1999; Grass et al. 2000; Munson et al. 2000; Saltikov and Olson 2002), precipitation as insoluble salts (Blake et al. 1993), immobilization within the cell wall (Cervantes and Gutierrez-Corona 1994), and production of chelating agents (Silver and Phung 1996; Lima et al. 2006a). The search for strategies that enhance metal tolerance, including cadmium, in bacteria probably helps to understand how this group of microorganisms copes with metal stress (Khan et al. 2009). In this context, Silver and Misra (1988) pointed out the importance of reevaluating the role of thiol in bacterial cell grown under Cd stress.

Glutathione (GSH) is a well-known thiol-containing tripeptide and a ubiquitous molecule with several roles in the cell metabolism such as reactive oxygen species scavenging, redox state regulation, transport of amino acids, and sulfur storage (Meister 1995; Noctor and Foyer 1998). GSH was reported to be important in acid, osmotic, and oxidative stresses (Chesney et al. 1996; Ferguson and Booth 1998; Riccillo et al. 2000). GSH is also one of the biomolecules with higher influence on heavy-metal tolerance in free-living rhizobia and in the nodulation and fixation processes (Harrison et al. 2005; Wani et al. 2007c). The addition of GSH to the growth medium increased Cd tolerance in yeast (Kang 1992), and it was central to

the survival of *Escherichia coli* under methylglyoxal exposure. This tripeptide was also proven to be essential to the survival of *Rhizobium tropici* under acidic conditions (Riccillo et al. 2000; Muglia et al. 2007). Furthermore, the ability to bind metals suggests its role as a detoxifying agent (Riccillo et al. 2000) and a key player in the tolerance to heavy metals in organisms that possess the GSH metabolic pathway.

Previous works (Figueira et al. 2005) reported that intracellular Cd levels were much higher in tolerant *Rhizobium leguminosarum* strains than in sensitive ones, when exposed to the same Cd concentrations. It was also proved that GSH levels were considerably increased in *Rhizobium* tolerant strains after Cd exposure but were not affected in sensitive ones. Moderately tolerant and tolerant strains presented higher intracellular GSH content under metal stress, being the highest GSH levels detected in the most tolerant strains (Figueira et al. 2005). From this study, it was concluded that the synthesis of this tripeptide could be related to Cd tolerance in *Rhizobium*. Therefore, it was our intention to assess the importance of GSH in the response of *R. leguminosarum* to Cd stress, showing that the events behind intracellular GSH increase are related to the mitigation of Cd toxicity and, consequently, enhanced the tolerance of *Rhizobium* strains to Cd stress. Taking this into consideration, exogenous GSH (80 μM) was added to YEM medium. Because Cd reacts with GSH, cells were first grown in YEM media supplemented with GSH for 5 h. Cells were then harvested by centrifugation and resuspended in an equal volume of fresh media, with or without Cd. Cadmium tolerance of *R. leguminosarum* strains was screened on YEM medium supplemented with different Cd concentrations (0, 0.25, 0.5, 0.75, and 1 mM). Although the Cd concentrations used in this study were higher than those observed in most of the contaminated ecosystems (Wagner 1993), the growth of microorganisms at stress levels that significantly affect their growth is a useful indicator for understanding the mechanisms of tolerance, since these are often triggered at high levels of stress.

Cadmium sensitivity was previously defined (Figueira et al. 2005; Corticeiro et al. 2006) as the inability of *Rhizobium* strains to grow on YEM medium added with Cd concentrations above 0.5 mM, while the tolerance of *Rhizobium* strains to Cd was defined as the ability to grow on YEM medium added with 1 mM Cd. Thus, strains CN-6 and NI-2 were considered sensitive to Cd, while strains M9 and NII-1 were moderately tolerant and E20-8 was tolerant. Figure 5.1a, b shows that sensitive rhizobial strains (CN-6 and NI-2) were unable to tolerate Cd concentrations above 0.5 mM and, at the lowest concentration (0.25 mM), presented a growth inhibition higher than 70% of control. The treatment with GSH, however, decreased the sensitivity of these strains to the metal, since at 0.25 mM Cd, growth increased and the highest Cd concentration tolerated by rhizobia enhanced to 0.75 mM Cd. In the moderately tolerant strains, M9 and NII-1 (Fig. 5.1c, d), GSH addition also increased the growth at all Cd concentrations tested, and the highest Cd concentration tolerated improved from 0.75 to 1 mM Cd. The tolerant strain E20-8 (Fig. 5.1e) was able to tolerate all Cd concentrations, but the addition of GSH to the growth medium also influenced the tolerance of this strain, as was evident by the enhanced growth at all Cd concentrations. These results supported the concept that within
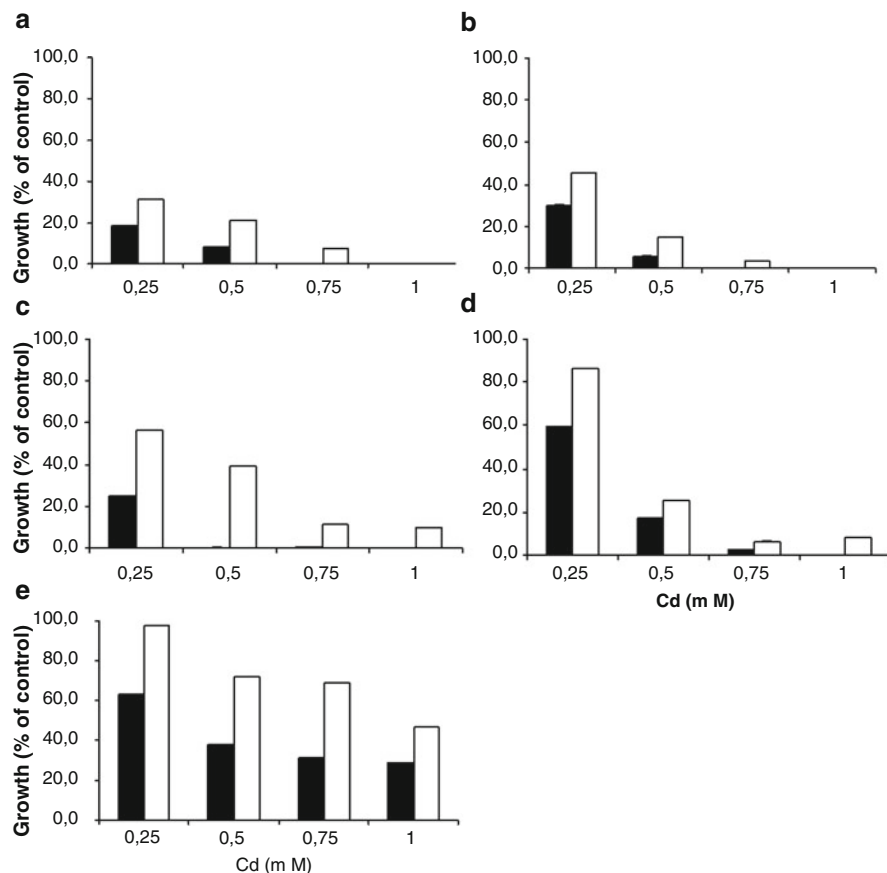
Fig. 5.1 Influence of GSH addition on the tolerance of *Rhizobium leguminosarum* to Cd. (**a**) CN6, (**b**) NI-2, (**c**) M9, (**d**) NII-1, and (**e**) E20-8. Growth in the absence (*black bars*) and presence (*white bars*) of GSH. Results are expressed as percentage of growth relative to controls (YEM medium not supplemented with Cd or GSH). Data are the means ± standard errors from three to eight replicate experiments

R. leguminosarum species, a high variability in Cd tolerance exists, as already reported (Purchase et al. 1997; Figueira et al. 2005; Pereira et al. 2006). The addition of exogenous GSH influenced Cd tolerance among all *Rhizobium* strains, indicating that the availability of this tripeptide was crucial for *Rhizobium* to cope with Cd stress. As reported earlier, for *R. leguminosarum*, GSH plays a dual function in Cd tolerance: protection of the cells from the oxidative stress induced by the metal (Corticeiro et al. 2006) and intracellular chelation of Cd (Lima et al. 2006a), protecting the intracellular metabolism from harmful effects of free Cd ions. The latter mechanism acts somehow similarly to that of phytochelatins (PC) in plants (Lima et al. 2006b), where free metal ions are sequestered in their SH moieties and open a new possibility for Cd tolerance in bacteria. Thus, GSH acts not only as an

antioxidant agent but also as a metal chelator. The addition of exogenous GSH to the growth medium allows sensitive strains, which did not have the ability to increase the synthesis of this tripeptide in the presence of Cd, to enhance growth under metal stress as well as to tolerate higher Cd levels. These results may be explained by higher intracellular GSH levels which may allow a more efficient Cd chelation (Lima et al. 2006a) and a higher scavenging activity of the reactive oxygen species induced by the metal, thus increasing the tolerance of *Rhizobium* strains to Cd.

5.4   Chronological Dependence of GSH Synthesis from Cd Intracellular Levels

Although GSH had a central role in *R. leguminosarum* tolerance to Cd, the chronology of this response to metal stress was not clear. Is it an early event? Or does GSH synthesis occur in a later stage of *Rhizobium* growth? The fate of the complexes formed is also unknown. In plant cells, PC–Cd complexes are sequestered in the vacuoles, but bacterial cells do not possess internal compartments. Thus, where are they accumulated? A metal-GSH exclusion system seems to be a plausible hypothesis. It is therefore crucial to monitor Cd uptake and GSH synthesis during *Rhizobium* growth in order to ascertain what triggers this mechanism of Cd tolerance. For this, two *Rhizobium* strains, with different Cd tolerance ability and GSH production capabilities, were chosen among the five strains previously investigated. Strains NI-2 (sensitive) and E20-8 (tolerant) were grown in YEM medium treated with or without Cd, and the intra- and extracellular Cd levels as well as GSH levels were monitored for 72 h. Due to marked differences in Cd tolerance, the strains were exposed to different Cd concentrations inducing identical growth inhibition (70%): 0.25 mM Cd for NI-2 and 1 mM Cd for E20-8.

In the absence of Cd, the growth of both rhizobial strains was identical; after a 3-h lag phase, exponential growth began until the 18th h, followed by a period of very slow growth up to the 72nd h. Under Cd exposure, the lag phase was extended up to 24 h for both strains and was followed by an increase in the growth rate up to 48 h for E20-8 and 72 h for NI-2 (Fig. 5.2). Results show that Cd exposure affected not only the number of cells at the end of the growth period (72 h) but also the dynamics of growth, leading to a longer lag phase, which could be indicative of a high bacterial metabolic effort to cope with Cd. These metabolic alterations seem to change the Cd uptake, which was very high in the first 12 h, decreasing afterward until the end of the growth period (Fig. 5.3a). The allocation of the Cd absorbed also varied over time (Fig. 5.3b, c). A high concentration of cadmium was retained in the cell walls during the first 24 h, while the intracellular Cd levels increased after the first day of growth, confirming cell wall retention as a primary defense system. These two events, reduction of Cd uptake and differential allocation of Cd in the cell, were present in both strains but were far more evident in the tolerant strain (E20-8) than in the sensitive one (NI-2). In control conditions, both strains synthesized similar levels of GSH that were partially excreted to the extracellular medium (Fig. 5.4a, b). In the presence of Cd, E20-8 efficiently restrained GSH
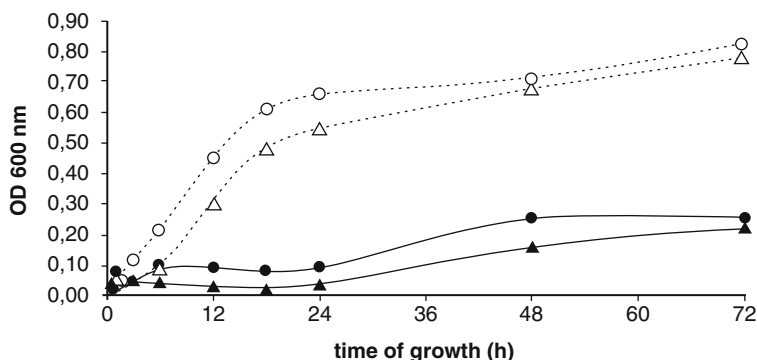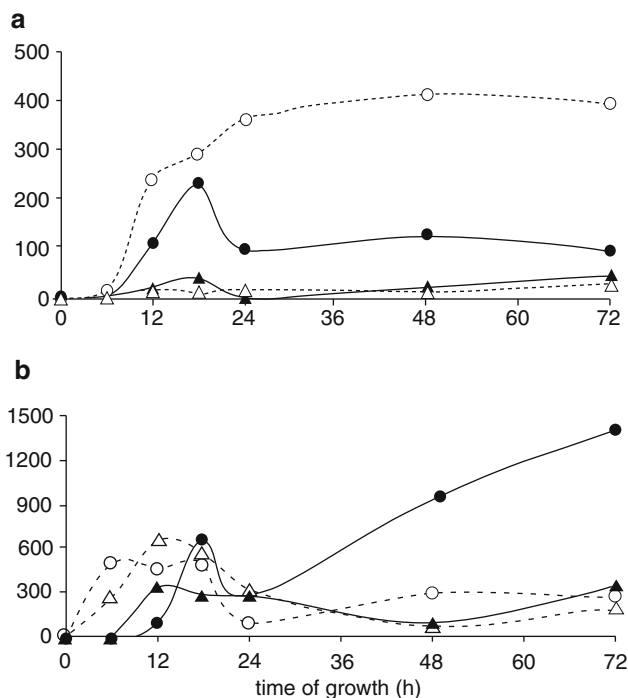
Fig. 5.2 *Rhizobium* growth in YEM medium with or without Cd. Growth of E20-8 in control conditions (*open circles*) and under 1 mM Cd (*black circles*) and of NI-2 in control conditions (*open triangles*) and under 0.25 mM Cd (*black triangles*). Data are the means from three to eight replicate experiments, with standard errors of less than 5%

exclusion. This event is in synchrony with the intracellular Cd increase that occurred after the first 24 h of growth. Hence, during the first 24 h, where Cd was retained mostly in cell walls, GSH seemed to be excreted to the extracellular medium, such as in control conditions. However, once Cd was accumulated in the intracellular space, where it could exert more damage, GSH was rapidly increased within the cell, scavenging reactive oxygen species and complexing Cd ions. These complexes are accumulated inside the cell and are not excreted to the medium, since no increment in extracellular GSH was detected. In strain NI-2, the intra- and extracellular concentrations of GSH were identical both in the presence and absence of Cd, indicating that this strain was not able to use efficiently GSH to tolerate Cd. Thus, a strong relationship between GSH synthesis and Cd tolerance triggered by the intracellular Cd concentrations suggests that GSH may play a critical role in the tolerance of *R. leguminosarum* to Cd, which, however, depends on the ability of cells to enhance the synthesis of GSH under Cd stress.

5.5 BNF and Plant Tolerance to Heavy Metals

Cadmium enters agriculture soils through both natural and anthropogenic processes. The average Cd concentration in soils worldwide is about 0.06 mg kg^{-1}, but some soils may contain Cd 10–1,000 times higher (He et al. 2005) than average concentration, of which more than 90% comes from anthropogenic sources (Pan et al. 2009). In severely polluted soils, Cd can be highly toxic to plants and soil microorganisms. Nevertheless, even subphytotoxic levels of Cd are of great concern due to its accumulation in food crops and consequently in food chain (Alloway 1995a). Pan et al. (2009), for example, demonstrated that food is the major route of Cd entry into the human populations. So, it becomes important to develop strategies that could help to avoid the metal uptake by legumes. In this context,
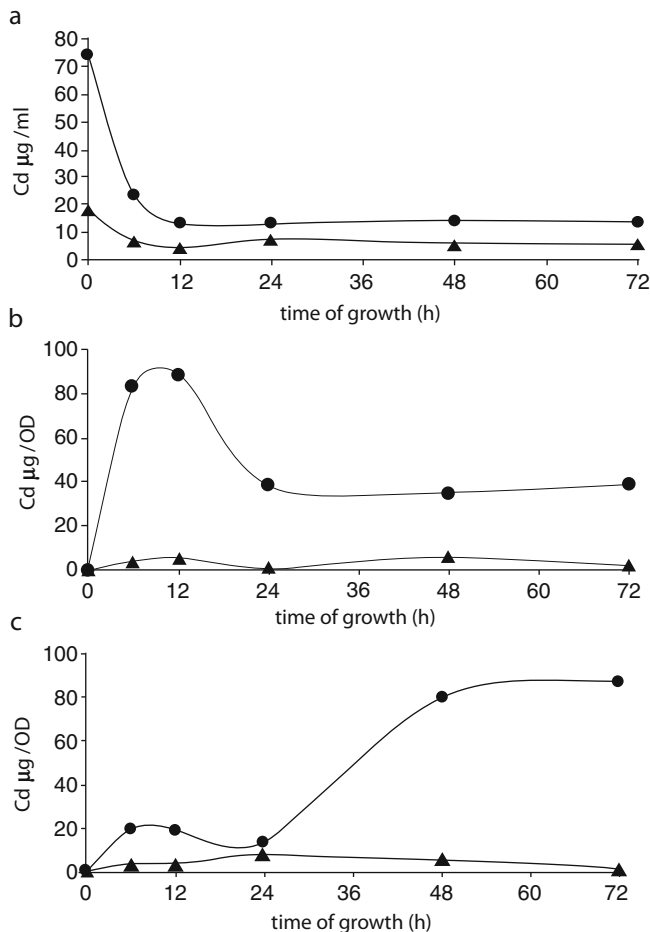
Fig. 5.3 Cadmium levels in E20-8 grown in 1 mM Cd (*black circles*) and of NI-2 in 0.25 mM Cd (*black triangles*). (**a**) Cd in the growth medium, (**b**) cell-wall-bound Cd, and (**c**) intracellular Cd. Data are the means from three to eight replicate experiments, with standard errors of less than 5%

nitrogen-fixing organs, like nodules, have been found to increase plants' survival to metals because bacteroids within nodules may counter metal stress by having a protective role against oxidative cell damage as observed in soybean (*Glycine max* (L) Merril) nodules (Balestrasse et al. 2001). Therefore, the research directed at understanding the mechanistic basis of metal tolerance by rhizobial strains while growing in contaminated soils is of great practical importance in different agroecological niches.

Conclusion

The higher degree of tolerance to cadmium was achieved only for strains able to induce GSH synthesis in the presence of high intracellular Cd levels.

Fig. 5.4 GSH levels in E20-8 in control conditions (*open circles*) and under 1 mM Cd (*black circles*) and of NI-2 in control conditions (*open triangles*) and under 0.25 mM Cd (*black triangles*). (**a**) GSH in the growth medium and (**b**) intracellular GSH. Data are the means from three to eight replicate experiments, with standard errors of less than 5%

Nevertheless, the phenotypic tolerance of rhizobial strains could be improved by exogenous GSH. Soil bacteria able to synthesize and excrete high levels of GSH may be applied to contaminated soil in consortia with the most effective strains in N$_2$ fixation, concomitantly improving an overall performance of legumes in heavy-metal-stressed soils.

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Bioremediation: A Natural Method for the Management of Polluted Environment

6

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Abstract

Heavy metal contamination resulting from rapid industrialization and other sources is a growing problem worldwide. Increasing pollution of soils with heavy metals disturbs the microbial biodiversity, soil fertility, and plant production and may cause significant human health problems. The excessive accumulation of heavy metals within plant tissues can modify protein structure or replace an essential element causing chlorosis, growth impairment, browning of roots, and photosystems dysfunction. To circumvent metal toxicity, bioremediation, a process that involves the use of biological materials to detoxify the contaminated sites and brings the environment to its contaminant free (original) state, has emerged as a promising alternative to widely practiced physicochemical methods used to clean up contaminated lands. Biological materials used to remediate contaminated sites are inexpensive, are easy to operate, do not produce hazardous by-products, and can be effective even if metals are present in low concentrations. Here, we integrate the knowledge obtained so far on the removal of metals and metalloids employing bioremediation strategies for contaminated soils. The information regarding different types of bioremediation and the challenges facing bioremediation are highlighted. The role and impacts of plant-growth-promoting rhizobacteria on bioremediation efficiency are addressed.

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6.1   Introduction

Heavy metals and metalloids are major global environmental pollutants, and many of them are toxic even at very low concentrations. For convenience, we will refer to both metal and metalloids as "metals" throughout the chapter. Such trace elements are released into the biosphere from different industries which use them frequently for manufacturing various products (Fernandes and Henriques 1991). The industries using heavy metals (HM) include mining, smelting, manufacturing, gas exhaust, energy and fuel production, fertilizer, sewage and pesticide production, and municipal waste generation. According to some estimates, metal concentrations in soil range from less than 1 mg kg^{-1} to as high as 100,000 mg kg^{-1}, which could either be geological in origin or may result from different human activities (Blaylock and Huang 2000). The bioavailability of heavy metals, however, depends on many factors, such as (1) environmental conditions, (2) pH, (3) species of element, (4) organic substances of the media, (5) fertilization, and (6) plant genotypes. Among the various industrial by-products, sewage, for example, which contains alarmingly high concentrations of toxic heavy metals (McGrath 1987) when applied in agricultural practices, is reported to have serious lethal impact on the viable and sustainable agroecosystem (Broos et al. 2004, 2005). For example, excessive accumulation of HM in plants including legumes such as green gram, pea, etc., has shown toxicity symptoms and has often been found detrimental as it (1) can modify the structure of some essential proteins (Yurela 2005; Roy et al. 2010); (2) can replace certain elements able to cause chlorosis (Ebbs and Uchil 2008), growth inhibition, structure damage, and browning of roots (Mahmood et al. 2007; Roy et al. 2010); and (3) can decline physiological and biochemical activities including inhibition of photosynthesis (Cheng 2003; Morita et al. 2006; Gorhe and Paszkowski 2006; Wani et al. 2007a; Ahmad et al. 2008; Babu et al. 2010). Apart from their effects on microbes and plants, upon ingestion of contaminated foods or drinking water, metals can be extremely dangerous for humans also.

The toxic nature of metals, their ability to persist in the environment and potential to contaminate agronomic soils, besides increasing demand for a steady, healthy food supply, therefore, requires urgent attention of the workers for making the environment safe from pollutants. Current management practices are based largely on the application of physicochemical approaches, which are often expensive and disruptive. Therefore, pressure on the scientists is mounting to identify and develop newer and safer strategies to replace or at least supplement the existing management options. In this regard, microorganisms of different origin and capable of facilitating plant growth through one or composite mechanisms, generally termed plant-growth-promoting rhizobacteria (PGPR), have been suggested to play a significant and vital role in alleviating the metal toxicity in different metal-contaminated soils (Khan et al. 2009; Jayabarath et al. 2009; Cardón et al. 2010; Cetin et al. 2011). The PGPR could detoxify the metal-contaminated environment by one or the simultaneous mechanisms acting together. These mechanisms are (1) the pumping of metal ions exterior to the cell, (2) accumulation and

sequestration of the metal ions inside the cell (Antony et al. 2011), (3) biotransfor-mation–transformation of toxic metal to less toxic forms (Cheung and Gu 2007; Shukla et al. 2009), and (4) adsorption/desorption of metals (Mamaril et al. 1997; Johnson et al. 2007). These mechanisms could be constitutive or inducive. The differences in the use of one or other metal removal/detoxifying mechanisms by microbes could however be influenced by the variations in the requirement, cell physiology, and the affinities of the bacterial cultures for the concentrations of metals present in different media. Irrespective of the mechanism adopted by PGPR for metal removal, the use of PGPR as seed/soil inoculants, in either conventional agriculture or metal-contaminated soils, has shown substantive increase in crop production and a noticeable decline in the toxicity of metals, leading to the enhancement in the growth and yield of chickpea (*Cicer arietinum* L.) (Gupta et al. 2004; Wani et al. 2008), greengram (*Vigna radiata* L. wilczek) (Faisal and Hasnain 2006; Wani et al. 2007b), tomato (*Lycopersicon esculentum*), Indian mustard (*Brassica campestris*), and canola (*Brassica rapa*) (Burd et al. 2000), grown in polluted soils. Use of PGPR strains endowed with many properties, like metal resistance/reduction ability (Joseph et al. 2007; Kumar et al. 2008; Wani and Khan 2010) and capacity to facilitate plant growth through variable mechanisms in metal-contaminated soils (Khan et al. 2009), is considered extremely important for the success of the bioremediation program. Briefly, PGPR promote plant growth by synthesizing or changing the concentration of plant growth enhancers like indoleacetic acid (Ahemad and Khan 2010a, b), gibberellic acid (Naz et al. 2009), cytokinins (Ortíz-Castro et al. 2008), and ethylene (Govindasamy et al. 2008; Duan et al. 2009), through asymbiotic N_2 fixation (Mirzaei et al. 2010), exhibiting antagonism against phytopathogenic microorganisms by production of siderophores (Wani et al. 2007a, b; Ahemad and Khan 2011a, b), antibiotics (Loper and Gross 2007; Jha et al. 2009), and cyanide (Devi et al. 2007; Rudrappa et al. 2008; Ahemad and Khan 2009), and solubilization of mineral phosphates and other nutrients (Khan et al. 2010; Ahemad and Khan 2011a, b).

6.2 Can Biotechnology Be Useful in Pollution Management?

Yes, biotechnology indeed can play an important role in shaping and preserving the agroecosystems by transforming the obnoxious pollutants into some benign products, generating biodegradable materials from renewable sources, and in the development of low-cost environmentally safe manufacturing and disposal methods. Environmental biotechnology, for example, while using genetic engineering, can improve the efficiency and could reduce the cost of microbial products used for different reasons including decontaminating the metal-contaminated soils. Considering the environment safety, researchers have suggested/applied various strategies collectively called bioremediation to rehabilitate areas poisoned by pollutants or damaged otherwise through ecosystem mismanagement.

6.3   Bioremediation: An Emerging Option

Bioremediation involves the use of living/dead organisms, to degrade/transform heavy metals into less toxic forms (Muller et al. 1996; Lloyd 2002; Memon and Schröder 2009). In the many forms of bioremediation (Fig. 6.1), microorganisms are utilized and managed through the control of environmental factors to reduce environmental pollution. Most bioremediation processes utilize indigenous microbial communities including PGPR (Khan et al. 2009), fungi (Zaidi et al. 2011), actinomycetes (El-Syed et al. 2011), algae (Huq et al. 2007), or plants (Marchand et al. 2010) to reduce, eliminate, contain, and transform metals of different origin to some benign products. Microorganisms used in bioremediation process could either be inhabitants of target contaminated sites or can be recovered from conventional soils (noncontaminated) and then applied into the metal stressed sites. The PGPR and symbiotic nitrogen fixers among microbes, for example, when applied, facilitate plant growth by several mechanisms and also play critical role in the restoration of damaged ecosystem (Khan 2004; Khan et al. 2009). Currently, scientists have directed their attention toward developing genetically engineered microbes for use in the field of bioremediation (Urgun-Demirtas et al. 2006; Singh et al. 2011).
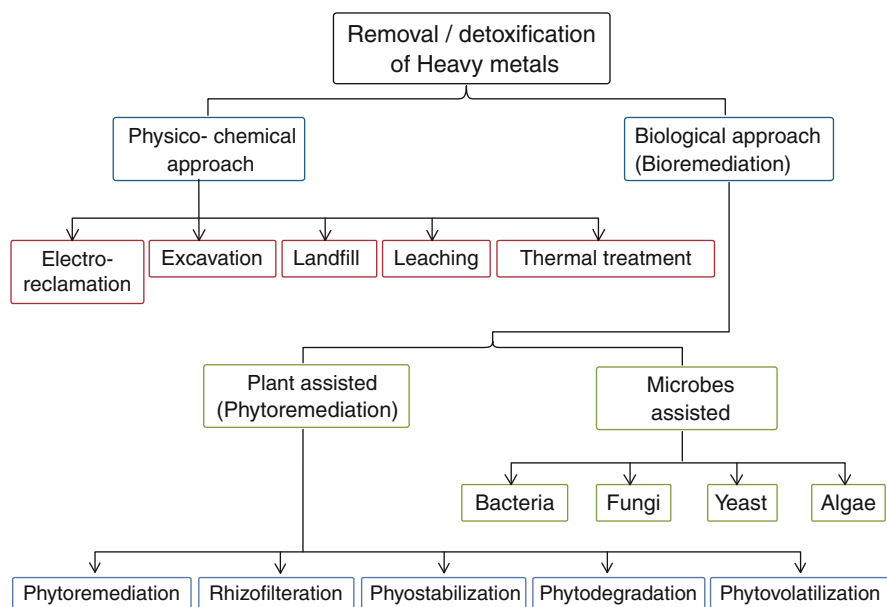
Fig. 6.1 Approaches used in the remediation of heavy metal toxicity from metal-contaminated site

6.3.1 Rationale for Using Bioremediation in Metal Decontamination

Being a natural process, bioremediation is easily accepted at global level as a reliable means for restoring the contaminated sites. Since the method involves the use of microbes/plants, this process is inexpensive compared to other physicochemical methods employed for removal/detoxification of metals from contaminated lands. This method can also be regularly applied for the contaminated site time and again without destructing soil properties. Due to this reason, the transport of waste from contaminated sites to the place of operation can be avoided which otherwise could lead to human health and the environment problems. In addition, when applied properly with sound understanding, bioremediation could be used to target many contaminants at one time resulting in the complete destruction of one or composite metals present in polluted soils. Despite these properties, bioremediation when practiced has certain dark side as well. For example, compounds which are nonbiodegradable in nature cannot be removed through this technology and, hence, persist in the environment. Moreover, since this method involves the use of biological materials, it is difficult to maintain optimum environmental condition suitable for growth of both microbial communities and plant genotypes, under field environment. Considering both the progress made in this direction and gaps in understanding the mechanistic basis of different bioremediation strategies, comprehensive efforts by the qualified and well-informed people from different disciplines are urgently required to fine-tune the bioremediation technologies so that these can be applied to a larger area of the world.

6.3.2 Types of Bioremediation

Bioremediation in general has been categorized into *in situ* and *ex situ* types (Table 6.1).

6.3.2.1 *In Situ* Bioremediation

In situ bioremediation is the application of biological treatment used to clean up the hazardous chemicals in the soil and surface or subsurface waters. This method, however, does not require excavation or removal of soils to accomplish remediation. This method is considered superior since it is a low-cost technology due in part to the use of renewable resources like microbes and causes minimal site disruption. Therefore, this method can be applied consistently for the contaminated sites. This method, however, also has certain problems like the following: (1) it is time-consuming compared to the other methods, (2) it is influenced by changes in environmental factors that are beyond human control, and (3) additives used in this method cause problems.

Table 6.1 Summary of bioremediation strategies

Technology	Examples	Benefits	Limitations	Factors to consider
In situ	*In situ* bioremediation	Most cost-efficient	Environmental constraints	Biodegradative abilities of indigenous microorganisms, presence of metals, environmental parameters, chemical solubility, geological factors, etc.
	Biosparging	Noninvasive	Extended treatment time	
	Bioventing	Relatively passive	Monitoring difficulties	
	Bioaugmentation	Natural attenuation process, treats soil and water		
Ex situ	Landfarming	Cost-efficient	Space requirements	Biodegradative abilities of indigenous microorganisms, presence of metals, environmental parameters, chemical solubility, geological factors, etc.
	Composting	Low cost	Extended treatment time	
	Biopiles	Can be carried out on site	Need to control abiotic loss, mass transfer problem, bioavailability limitation	
Bioreactors	Slurry reactors Aqueous reactors	Rapid degradation kinetics, optimized environmental parameters, enhanced mass transfer, effective use of inoculants and surfactants	Soil requires excavation, relatively expensive	Bioaugmentation, toxicity of amendments, toxic concentration of contaminants

Adapted from Vidali (2001)

6.3.2.2 *Ex Situ* Bioremediation

This type of bioremediation requires excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation. This technique has more disadvantages than advantages.

6.3.3   Some Examples of Bacteria-Mediated Bioremediation

The ability of microbial communities to transform certain natural and synthetic chemicals into the products which could later on be used as sources of energy and raw materials for their own growth is one of the unique characteristics of microbes. Because of this important trait, microbes have been considered as a good alternative to various existing chemical or physical remediation processes and are less expensive (Chen et al. 2005; De et al. 2008). Even though microorganisms have been found promising in remediation technology, majority of them inhabiting different agroecological systems have yet not been explored in environmental biotechnologies. Therefore, consistent effort is needed to identify efficient

microorganisms for bioremediation purposes. In this direction, several attempts have however been made, some of which have been successful. For example, *Geobacter metallireducens* has been found to remove uranium, a radioactive waste, from drainage waters in mining operations and from contaminated groundwaters (Magnuson et al. 2000; Lloyd and Lovley 2001). Even dead microbial cells can be useful in bioremediation technologies. These discoveries suggest that further exploration of microbial diversity is likely to lead to the discovery of many more organisms with unique properties useful in bioremediation. In one study, Lee et al. (2006) for example found that *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D could accumulate higher concentration of cadmium in the presence of 16 mM CdCl$_2$. In another study, Wani and Khan (2010) reported that the *Rhizobium* could bioremediate chromium when grown in chromium-amended medium. In a similar study, Pan et al. (2009) observed that *Penicillium* and *Fusarium* biosorbed lead while Ting and Choong (2009) observed that *Trichoderma* bioaccumulated and biosorbed other heavy metals. In another study, Yan and Viraraghavan (2003) also analyzed the biosorption capacity of *Mucor rouxii* for different heavy metals in binary and ternary solutions. The efficacy of using bacterial biomass for sequestering heavy metals is well documented. The selective adsorption of Pb, Cu, and Cd with fixed-bed columns containing immobilized bacterial biomass was 25–30% more efficient than columns packed with a chemical matrix (Chang and Huang 1998). In another example, it was discovered that *Pseudomonas aeruginosa* can accumulate nickel as its phosphide and carbide crystal in the cell envelope region (Sar et al. 2001). It has been discovered that the filamentous fungus *Rhizopus arrhizus* can be used for biosorption of Pb ions from storage battery industry wastewaters, and it can remove 2.643 mg Pb(II) g^{-1} microorganism (Bahadir et al. 2007).

6.3.4   Bacteria-Assisted Phytoremediation

Generally, plants including legumes like chickpea (Wani et al. 2008b; Wani and Khan 2010), lenti (Wani et al. 2006), greengram (Kumari et al. 2011), *Allium stivum* and *Vicia faba* (Unyayer et al. 2006), pea (Wani et al 2008a), etc., are susceptible to heavy metal toxicity. The toxicity, however, depends largely on (1) the concentration and types of metal and (2) plant genotypes, stage of plant growth, and their metal uptake ability. On the other hand, plants also respond to heavy metals differently to avoid deleterious effects in a variety of ways. For example, tolerance to metals is based on one or multiple mechanisms like cell wall binding (Kang et al. 2007), active transport of ions into the vacuole (Salt and Rauser 1995), and formation of complexes with organic acids (Delhaize and Ryan 1995) or peptides (Kotrba et al. 1999). The other most widely recognized mode of metal detoxification in plants appears to be chelation of metals by low-molecular-weight proteins such as metallothioneins (MT) (Kille et al. 1991) and peptide ligands, the phytochelatins (Grill et al. 1985). For example, glutathione (GSH), a precursor of phytochelatin synthesis, has been reported to play a vital role in metal detoxification

(Maitani et al. 1996) and in protecting plant cells from other environmental stresses including intrinsic oxidative stress reactions.

Besides plants, many PGPR inhabiting rhizosphere have been found to play significant roles in mobilization or immobilization of heavy metals (Gadd 1990; Rajkumar and Freitas 2008) and consequently reduce the availability of metals to plants. This in turn indirectly protects plants from metal toxicity. However, only very few attempts have been made to identify rhizosphere bacteria with metal-accumulating ability and plant colonizing potential which could be of practical importance in alleviating metal toxicity when inoculated plants are grown in metal-contaminated soils. Thus, the ability of plants to remove/sequester metals in contaminated sites, generally called phytoremediation, can be improved by applying PGPR simultaneously with various phytoremediation methods, such as (1) phytoextraction (phytoaccumulation), (2) rhizofiltration, (3) phytostabilization, (4) phytodegradation (phytotransformation), (5) rhizodegradation, and (6) phytovolatilization, used for removing metal toxicity from contaminated lands (Denton 2007; Abou-Shanab 2011). Phytoremediation as a technique is inexpensive since it involves plants which can be easily grown and monitored (Saraswat and Rai 2011). Moreover, the recovery and reuse of valuable products in this method are easy because they use natural biological materials, plants can be modified for any target characteristics, and the original state of the environment could be restored. Among the disadvantages, phytoremediation is quite often a lengthy process and affected greatly by the changing environmental conditions. However, when applied as inoculants along with the phytoremediation process, PGPR also affect the mobility and availability of metals to plants by releasing numerous chelating substances, acidification, phosphate solubilization, and redox changes (Whiting et al. 2001). In addition, siderophores released by PGPR including legume-nodulating rhizobia (Wani et al. 2008b; Ahemad and Khan 2011a, b) into the rhizosphere serve as an iron source for plants (Burd et al. 2000) and therefore help to fulfill the iron deficiency of plant in iron-deficient soils. It is therefore believed that the best way to prevent plants from metal toxicity was to use PGPR capable of producing siderophore bacterium. For example, Burd et al. (1998) showed that PGPR, when applied to soils, increased the growth of plants even in the presence of metals like Ni, Zn, and Pb and allowed the plants like tomato, Indian mustard, and canola inoculated with *Kluyvera ascorbata* to develop larger roots and get better established during early stages of growth (Burd et al. 2000). To validate this further, three heavy-metal-resistant PGPR, such as *P. putida* strains *and P. fluorescens* strains able to produce indole-3-acetic acid (IAA), siderophores, and 1-aminocyclopropane-1-carboxylic deaminase (ACCD), were used to inoculate canola and barley seeds in a soil artificially contaminated with $CdCl_2$ (10 and 20 mg kg^{-1}) and $Pb(No_3)_2$ (300 and 600 mg kg^{-1}) in a pot experiment. The inoculated canola plants had maximum shoot dry matter. In addition, there was an increase in Cd and Pb uptake by canola plants inoculated with *PGPR strains*. Furthermore, the translocation factor indicated that inoculated canola and barley had abilities of Cd and Pb phytoextraction in the contaminated soil, respectively. Therefore, overall improvement in the inoculated canola and barley plants was suggested due to the protection against the inhibitory effects of cadmium and lead by PGPR in addition to

their ability to provide IAA, siderophore, and ACCD to the developing plants (Yancheshmeh et al. 2011). In a similar study, green gram plants, when inoculated with *Ochrobacterium intermedium* and *Bacillus cereus,* were protected from chromium toxicity (Faisal and Hasnain 2006). More recently, *Lupinus luteus* plants, inoculated with metal-resistant rhizobacteria, *Serratia* sp. MSMC541, was used to assess its effect on the phytostabilization of metals in contaminated soils (El Aafi et al. 2012). The strain MSMC541 showed resistance to several metals up to 13.3 mM As, 2.2 mM Cd, 2.3 mM Cu, 9 mM Pb, and 30 mM Zn. Also, strain MSMC541 could biosorb great amounts of metals in cell biomass. When tested in pot trials, strain MSMC541 improved the *L. luteus* tolerance to metals by significantly reducing the metal translocation to the shoot, suggesting a greater role of Serratia sp. MSMC541 inoculated *L. luteus* plants in phytostabilization of metal-contaminated soils. The other mechanism by which PGPR improves growth and yield of crops includes the accumulation of potentially toxic trace elements into plant tissues and subsequent reduction of metal toxicity by absorbing/adsorbing them (Mamaril et al. 1997). As an example, accumulation and concomitant reduction in metal toxicity have been reported for *O. intermedium*-inoculated sunflower (*Helanthus annus*) grown in chromium-polluted soils. Considering the various characteristics, the overall stimulation/increase in the growth of crops due to PGPR inoculation in metal-contaminated soils could be due to the ability of PGPR strains to (1) exhibit high level of tolerance against varying concentration and different types of metals, (2) synthesize and release growth enhancers in rhizosphere, and (3) transform toxic metals to less toxic forms. Based on the data obtained and understanding of these complex subjects, it is suggested that PGPR strains possessing such vast properties could be used as inoculants for enhancing the growth of plants in soils contaminated with toxic metals. However, before they are recommended for field application, more and more field trials are required in areas contaminated with metals.

Conclusion

The remediation of metal-enriched soils employing biological systems such as microbes or plants is an interesting but emerging area which has shown considerable progress in different agroecological regions. Further development of bioremediation involving microbe-assisted phytoremediation, however, still requires an integrated multidisciplinary research effort that should include plant biologists, molecular biologists, soil chemists, and microbiologists together with agronomists and environmentalists. Thus, the combined efforts of various groups are likely to achieve greater success in alleviating the toxicity from heavy metal-contaminated sites. Furthermore, constructing microbes/plants using genetic engineering for specifically chosen characteristics like adapting well to local environmental conditions, expressing high metal tolerance, or increasing metal uptake capacity of both partners (microbes and plants) is likely to increase the efficiency of bioremediation and makes the bioremediation more viable, eco-friendly, and probably an undeniable technology. The major challenges associated with release of genetically engineered biological

materials in field conditions, however, requires a careful attention. Besides scientific developments, constant monitoring and regulation of government agencies over the problems of heavy metal pollution and broad participation of society in this campaign are essentially warranted.

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Rhizobium–Legume Symbiosis: A Model System for the Recovery of Metal-Contaminated Agricultural Land

Santi M. Mandal and Rabindranath Bhattacharyya

Abstract

Legumes are considered the appropriate crops for raising the productivity and recovery of marginal lands through symbiosis with nodule-forming bacteria collectively called rhizobia. Cultivated fields around the world including India are often irrigated by metal-contaminated groundwater and surface water. This practice poses a significant risk to both agroecosystems and human health via food chain. Therefore, metal removal from contaminated soils is urgently required. In this context, conventional technologies for metal removal have been employed, but they are expensive and disruptive. The use of biological materials including both plants (phytoremediation) and microbial communities in the remediation of polluted environments, on the contrary, has been found environment friendly and inexpensive. Leguminous plants have been found important in this regard due to their bioremediation potential and ability to provide essential nutrient nitrogen to plants in nitrogen deficient soils through symbiosis with rhizobia. The role of *Rhizobium*–legume symbiotic association in alleviating metal toxicity is reviewed and highlighted.

7.1 Introduction

Legumes play an important role in improving soil fertility and are, therefore, introduced to newer areas to enrich soil N pool. In addition, legumes serve as an important source of dietary protein, and hence their production is linked to food security. The other partner of legume–*Rhizobium* symbiosis, the rhizobia in general, is Gram-negative soil bacteria that have a profound scientific and agronomic significance due to their ability to establish nitrogen-fixing symbiosis with legumi-

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nous plants and consequently serve as major source for maintaining soil fertility (Yates et al. 2008). During *Rhizobium*–legume symbiosis, the nodule formed on the root systems of legume is a unique and highly organized structure within which the invading bacteria (*Rhizobium*) differentiate into nitrogen-fixing bacteroids that provide reduced N to the plant in exchange for carbohydrates and shelter (Schauser et al. 1999; Becker et al. 1998; Kouchi et al. 2010). A successful *Rhizobium*–legume symbiosis thus is the most prominent plant–microbe endosymbiotic system and has critical importance in agriculture which is likely to increase the N pool of soil ecosystems. *Rhizobium*–legume symbioses are the primary source of fixed N in land-based systems and provide well over half of the biological source of fixed N (Tate 1995). According to some estimates, biological nitrogen fixation (BNF) can provide 200–300 kg of N ha^{-1} $year^{-1}$ to soils (Peoples et al. 1995). However, the measured amounts of N fixed by symbiotic systems may differ according to the method used to study N_2 fixation (Sellstedt et al. 1993). For example, fixed N added to terrestrial ecosystems resulting from the symbiotic relationship between legumes and their corresponding specific rhizobial species has been found as 70 million tons of N per year (Brockwell et al. 1995). The symbioses between *Rhizobium* and legumes are however a cheaper and usually more effective and attractive agronomic practice for ensuring an adequate supply of N than the application of synthetic N fertilizer. To substantiate this, Mandimba (1995) has revealed that the N supplied by *Arachis hypogaea* to maize (*Zea mays*) plants in intercropping systems was equivalent to the application of 96 kg of fertilizer N ha^{-1}.

The establishment of an effective nitrogen-fixing *Rhizobium*–legume symbiosis depends on several factors like (1) efficacy of rhizobial strains to fix atmospheric nitrogen, (2) the survivability of the microsymbiont both within nodules and outside host in soils, and (3) the ability of host to fulfill the needs of the bacteria once a nodule is formed (Freire 1984). These variables are influenced by several limiting factors found in soil viz. moisture content, aeration, pH, heavy metals, and soil nutrients. All of these can have a direct effect on the viability of the free-living rhizobia (Pereira et al. 2006a; Chaudri et al. 2008; Stan et al. 2011), the host plant (Wani et al. 2007), and/or the symbiosis (Younis 2007) itself. Moreover, interactions between the limiting factors may produce a greater negative impact than the effect of individual factors. As an example, excessive heavy metal content greatly influences the survival of rhizobia (Paudyal et al. 2007) and the health of the legumes (Bordeleau and Prevost 1994; Wani et al. 2008a, b). A major part of the cultivated field throughout the world is also contaminated with heavy metals which indirectly affects human health through food web (Chaterjee et al. 1995; Das et al. 2004) because heavy metals are nondestructive and persist in soil (Khan et al. 2009). The *Rhizobium*-inoculated legumes grown in various production systems with diverse cultural practices carry traits that have allowed them to adapt to the adverse environmental conditions (Carrasco et al. 2005). This has been found largely due to the ability of rhizobia to tolerate higher concentration of metals in soils polluted with heavy metals (Yang et al. 2005; Mandal et al. 2008). Besides this, a number of heavy-metal-accumulating plants (Ma et al. 2001;

Franceseoni et al. 2002) have also been reported but have been found of limited use because of their poor ability to produce biomass and slow growth rate. Considering the multiple activities, *Rhizobium*–legume symbiosis has been found as an alternative to physicochemical methods for removing/reducing metal toxicity from contaminated sites (Sprent 1997).

7.2 Source of Heavy Metals in Agricultural Fields

Heavy metal pollution is one of the major problems that adversely affect human health. Environmental contamination due to anthropogenic and natural sources is increasing because of consistently increasing human populations, industrialization, and urbanization. As a result of these factors, a great number of pollutants and waste materials containing heavy metals are disposed off into the environment. Uptake and subsequent accumulation of heavy metals in crops grown in metal-polluted soil leads to poor or no growth and may alter symbiosis leading consequently to the yield loss (Moftah 2000; Wani et al. 2007). The consumption of such crops in turn severely affects human health (Fu et al. 2008).

The primary source of heavy metal pollution in soils is the parent materials from which the soils are formed. However, the influence of parent materials on the total concentrations and forms of metals vary with pedogenic processes (Herawati et al. 2000). Industrial operations such as smelting, metal forging, and combustions of fossil fuels, etc. are other source of metal pollution (Khan et al. 2009). There are yet other sources of metal contamination in mining areas which include grinding, concentrating ores, and tailings disposal (Adriano 1986; Wang et al. 2004). Inappropriate treatment of these tailings and acid mine drainage could pollute the agricultural fields surrounding the mining areas (Williams et al. 2009). Fly ash also adds significant amounts of metals to the soil environment (Liu et al. 2006).

Agricultural uses of pesticides are also another source of heavy metals in cultivated fields. Pesticides containing cadmium, mercury, lead, copper, zinc, and other trace elements have been reported. The industrial effluents often contain many heavy metals. In industrial areas, many agricultural fields are flooded by mixed industrial effluents or are irrigated with treated industrial waste water (Sinha et al. 2006). Agricultural land is irrigated by waste water in several developing countries. Though, sewage irrigation can overcome the water shortage to some extent, but it can also add some heavy metals to soils and, concomitantly, cause serious environmental problems. Many industrial plants routinely discharge their waste into drains, which either contaminate rivers and streams or add to the contaminant load of biosolids (sewage sludge). Biosolids are increasingly being used as soil ameliorants and streams and rivers are the primary source of water for irrigation (McGrath 1994). Amending agricultural land with biosolids is a common practice that alters the physiochemical properties of soil (Obbard et al. 1994). Biosolids may contain excessive quantities of heavy metals that may persist in soil long after application. Sewage sludge is applied to agricultural land both as a means of sewage disposal and to recycle plant nutrients.

Of the various heavy metals, some heavy metals, like Fe, Mo, and Mn, are important as micronutrients; some toxic heavy metals like Zn, Ni, Cu, V, Co, W, and Cr have roles as trace elements, and there are some heavy metals, for example, Hg, Ag, Cd, Pb, and U, whose nutritional functions are not known but are nonetheless toxic for plants and microorganisms (Schutzendubel and Polle 2002). In addition, there are also a number of metalloids including arsenic that are highly toxic for human beings. In recent years, the surface layer of the agricultural field has been found highly contaminated by the excessive use of pesticides including, fungicides, insecticides, heavy metals, and arsenic through irrigation. Many people have died and hundreds of millions are at serious risk in many countries such as Bangladesh, India, China, Vietnam, Taiwan, Japan, Poland, Hungary, Romania, Slovakia, Belgium, Chile, Argentina, and North Mexico (Niu et al. 1995; Chowdhury et al. 1999). Arsenic is accumulated in the soil in a number of ways like during copper and lead smelting and different chemical manufacturing process; factories that produce pesticides, herbicides, and other agricultural products are exposed to arsenic (Klaasen and Watkins 2003). Another important source of arsenic contamination is through irrigation of land by groundwater. Excessive drawing of groundwater leads to the release of arsenic from its core compound called arsenopyrites by oxidation in contact with air (Mandal et al. 1996). It is reported that groundwater arsenic level of nine districts of West Bengal, India, is $50 \, \mu g \, l^{-1}$, which is above the standard maximum permissible limit by World Health Organization (Chowdhury et al. 1999). This groundwater is used for irrigating agricultural fields, thereby enhancing the human risk of arsenic poisoning through the food chain as well (Das et al. 2004).

7.3   Mechanisms of Heavy Metal Resistance in Rhizobia

Rhizobium–legume symbiosis is affected by numerous environmental factors including pH, oxidation–reduction potential, aeration, clay minerals, and metal oxides. Some of these factors enhance toxicity of metals that ultimately affect soil microbiota. Numerous studies have found negative effects of heavy metals from biosolids-amended soils on indigenous populations of many rhizobia, like *Rhizobium leguminosarum* bv. trifolii (Giller et al. 1998), as well as positive effects on microsymbionts of other legumes (Heckman et al. 1987; Kinkle et al. 1987). There is however a major concern that heavy metals may have long-term detrimental effects on soil microorganisms and may change microbial composition of soils (Juste et al. 1995), nodulation, and nitrogen fixation in leguminous plants (Purchase et al. 1997). It has also been reported that *Rhizobium leguminosarum* bv. *trifolii* isolated from sludge-treated soil could induce nodulation in white clover (*Trifolium repens* L. Blanca) but was ineffective in nitrogen fixation (Ibekwe et al. 1995). The potential for the *Rhizobium*–legume symbiotic association in metal-contaminated soils to fix nitrogen depends upon the effective and metal-resistant rhizobial population in soil. To survive under metal-stressed conditions, bacteria have evolved several mechanisms to tolerate the uptake of heavy metal ions (Fig. 7.1).
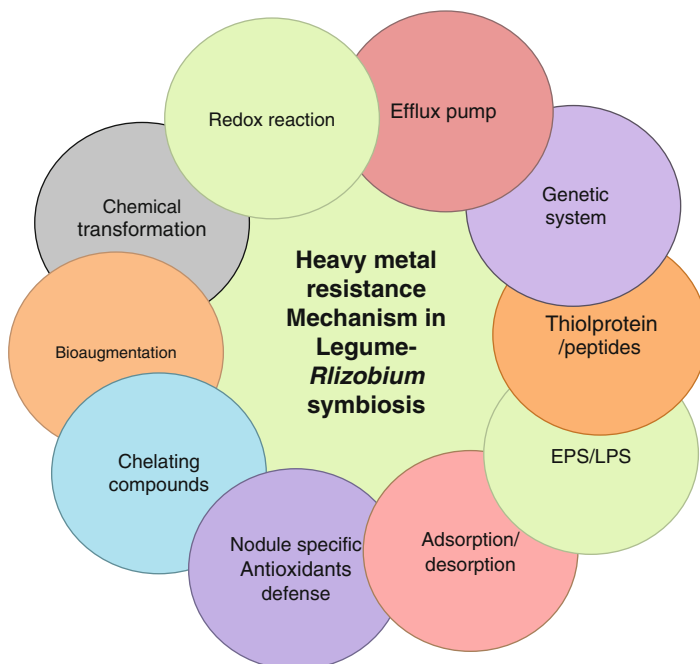
Fig. 7.1 Schematic diagram of heavy-metal-resistant mechanism in legume–*Rhizobium* symbioses

These mechanisms include precipitation of metal as insoluble salts by chemical transformation (Blake et al. 1993), energy-dependent efflux of metal (Nies 1992), production of chelating agents (Ow 1993), and sometimes metal resistance is plasmid-mediated and biochemical transformation of metal ions (Silver and Walderhaug 1992). Metal ions are known to cause oxidative stress by the Fenton reaction, and while there is some knowledge as to how rhizobia counter oxidative stress, there is little known on that caused by heavy metals (Balestrasse et al. 2001). Stress response genes are induced as metal ion concentrations increase from starvation to toxic levels. It has been shown that there are genes that are expressed under specific metal stress (Singh et al. 2001). Interestingly, the acid tolerance gene *actA* is required in *Sinorhizobium meliloti* to develop copper and zinc resistance, though it is not resolved completely (Tiwari et al. 1996a). Mutations in the acid-induced genes *actA*, *actR*, or *actS* are sensitive to copper and zinc (Reeve et al. 2002). A direct correlation has been established previously (Keyser and Munns 1979; Tiwari et al. 1996b), and an acid-induced copper pump, *ActP*, has also been found in *S. meliloti* which is controlled by a heavy-metal-responsive regulator (HmrR) (Reeve et al. 2002). Copper and zinc also activate the PhrR repressor (Reeve et al. 1998). A number of microorganisms are capable of using either the oxidized form of inorganic arsenic As (V) or the reduced form As (III) in their metabolism and even more microorganisms are capable of resisting arsenic toxicity

through the *ars* genetic system (Oremland and Stolz 2003). To resist the arsenate invasion, some microorganisms have developed or acquired genes that permit the cell to neutralize the toxic effects of arsenic through the exclusion of arsenic from the cells via *ars* operon. Arsenic resistance genes, namely, *arsC*, have been identified in *Rhizobium* species (Mandal et al. 2008). Identification of the *arsA* gene in *Mesorhizobium loti* confirms the presence of an *ars* operon and consequently arsenate resistance (Sá-Pereira et al. 2007).

Rhizobium has the ability to produce a huge amount of extracellular polysaccharide (EPS) and lipopolysaccharide (LPS) (Mandal et al. 2007). Both LPS and EPS sequestrate most of the metal extracellularly, acting as a first-defense barrier against heavy metal stress. However, EPS or LPSs were not enough to support the highest levels of stress imposed, probably because binding sites may be saturated, allowing ions to enter into the cell and raising the concentrations inside the cell, particularly in extremely tolerant isolates (Pereira et al. 2006b). Responses to some of these metals have been characterized, for example, high intercellular carbohydrates and large cell inclusions increase the resistance of *R. leguminosarum* to cadmium, copper, nickel, and zinc. Furthermore, production of thiols has also been shown to counter heavy-metal-induced oxidation (Balestrasse et al. 2001). Thiols bind to the metal ions, forming a complex and preventing any cell damage by inactivating the ion's redox potential and have been shown to be effective against cadmium, gold, mercury, and lead toxicity (Singh et al. 2001). In a follow-up study, Lima et al. (2006) demonstrated that GSH–Cd chelation is a novel-induced mechanism of Cd detoxification in *R. leguminosarum*. Nodules can help plants survive because the bacteroids counter metal stress and supporting the symbiosis is mutually beneficial to legume and rhizobia.

7.4   *Rhizobium*–Legume Symbioses as Phytoremediator

The generic term "phytoremediation" consists of the Greek prefix phyto (plant), attached to the Latin root remedium (to correct or remove an evil) (Cunningham et al. 1996). This technology can be applied to both organic and inorganic pollutants present in soil (solid substrate), water (liquid substrate), or the air (Raskin et al. 1994; Salt et al. 1998). Phytoremediation, defined as the use of vegetation for in situ treatment of contaminated soils, sediments, and water, is an environmental biotechnology that has attracted recently the interest of scientists, public opinion, regulators, and public administration. The physicochemical techniques for soil remediation 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 during the decontamination process. Phytoremediation consists of six main processes: (1) phytotransformation, ideal for organic contaminants in all substrates, (2) rhizoremediation, applied to organic contaminants in soil, (3) phytostabilization, for organic and inorganic contaminants in soil, (4) phytoextraction, useful for inorganic contaminants in all substrates, (5) phytovolatilization, which concerns volatile substances, and (6) evapotranspiration,

to control hydraulic flow in the contaminated environment. All phytotechnologies can be applied only if the contaminant is in contact with roots, and most of them rely on contaminant uptake by roots. Metals must be taken up into plants and transported to the shoots and leaves despite their possible toxicity. Moreover, the work of a number of researchers indicates that adding the soil bacteria can facilitate this process. Thus, eventual successful commercialization of metal phytoremediation is likely to include a more complete understanding of the role of bacteria in this process and to harness their synergistic potential for promoting plant growth and metal uptake in metal-contaminated soils. Plants can be used to accumulate inorganic and organic contaminants, metabolize organic contaminants, and encourage microbial degradation of organic contaminants in the root zone. Widespread utilization of phytoremediation can be limited by the small habitat range or size of plants expressing remediation potential and insufficient abilities of native plants to tolerate, detoxify, and accumulate contaminants.

There is plethora of information available on heavy-metal-accumulating plants (Ma et al. 2001; Franceseoni et al. 2002), but the application of such plants in metal remediation is limited due to the ability of plants to produce small biomass, slow growth rate, and some unknown agronomic potential. However, the use of leguminous plant in metal removal/detoxification from contaminated sites is beneficial both ecologically and agronomically since it is a major source of BNF which provides sufficient amounts of N to developing legumes (Sprent et al. 1987). Thus, legumes and rhizobia are often desirable species during, and after, the remediation of heavy-metal-contaminated land.

7.5 Importance of Recombinant Rhizobia in Heavy Metal Bioremediation

Higher organisms respond to heavy metals by producing metallothioneins (MTs). Metallothioneins are low-molecular-weight cysteine-rich peptides capable of high affinity coordination of heavy metal ions via cysteine residues shared along the peptide sequence in Cys–X–Cys or Cys–Cys motifs that bind various HMs. For example, *Saccharomyces cerevisiae* CUP1 with 12 cysteine residues forms a 53 amino acid MT variant having eight binding centers for monovalent and four binding centers for divalent heavy metal ions. In these organisms, the intracellular sequestration of toxic heavy metal ions via MTs represents one of the principal mechanisms conferring tolerance to particular heavy metal ions (Kotrba et al. 1999; Vašák 2005).

Overproduction of recombinant MTs in order to enhance resistance to HMs and support metal accumulation in plants or bacteria may be an attractive approach. In this context, bacteria with the high metal-binding capacity of MTs have been widely reported and exploited (Ji and Silver 1995). Intracellular expression of MTs does not avoid the complications, and in many instances there have been problem with the stability and short half-life of the expressed heterologous proteins. This is due to the high cysteine content of MTs, which might interfere with cellular

redox pathways in the cytosol (Raina and Missiakas 1997). Earlier human tetrameric metallothionein (MTL4) has successfully overproduced the protein in *E. coli* (Hong et al. 2000). The overproduced tetrameric MTL4 bound 28 g atoms of Cd or Zn, whereas the human monomer MT bound 7 g atoms of Cd or Zn per one MT molecule (Hong et al. 2000). Recently, MTL4 has been engineered into a more environmentally robust bacterium such as *Rhizobium* which can successfully infect legume and lead to the formation of a nitrogen-fixing nodules on the root of legume, containing over 10^8 bacterial progeny (Downie 1997). This special character is useful for biotechnological application for the expression of genes such as metallothionein that sequesters HMs from contaminated soil. Once symbiosis is established, the HMs will be accumulated in nodules. This is an alternative and less expensive method to remove HMs from the soil.

Other than MTs, phytochelatins (PCs) are small peptides (γ-Glu–Cys)$_n$ X (PCn; $n = 2$–11; X represents Gly, Ser, β-Ala, Glu, Gln, or no residue) found in plants and in certain yeasts. These peptides are capable of an efficient sequestration of multiple metal and metalloid ions in metal (loid)–thiolate complexes and play a vital role in heavy metal detoxification in plants (Clemens 2006; Cobbett and Goldsbrough 2002). Unlike gene-encoded MTs, PCs are enzymatically synthesized in a transpeptidation reaction from glutathione (γ-glutamylcysteinylglycine, GSH) or its homologues (iso-PCs) by the constitutive PC synthase (PCS) in a metal- or metalloid (e.g., arsenate)-dependent manner. The low-molecular-weight metal–PC complexes of 2–4 kDa formed in cytosolic compartment could be further transported to vacuoles where immobile 6–9 kDa high-molecular-weight complexes of metal sulfide crystallites covered with PC are formed under the incorporation of S^{2-} (Clemens 2006; Kotrba et al. 1999).

A research group of Yamashita developed a bioremediation system based on the symbiosis between *Astragalus sinicus* and *Mesorhizobium huakuii* subsp. rengei B3, which has been isolated as the specific bacterium (Murooka et al. 1993; Nuswantara et al. 1999). It established a symbiotic relationship with *A. sinicus*, the legume that has been used as green manure in rice fields in China and Japan, by eliciting formation of nitrogen-fixing root nodules (Chen et al. 1991). The genes encoding synthetic tetrameric metallothionein (MTL4) (Hong et al. 2000) and phytochelatin synthase from *A. thaliana* (AtPCS) were cloned under the nolB (Ruvkun et al. 1982; Perret et al. 1999; Sriprang et al. 2002) or nifH (Meinhardt et al. 1993; Freiberg et al. 1997; Sriprang et al. 2003) promoter, which generated nodule specific expression of these genes, and introduced into *M. huakuii* subsp. *rengei* B3. These two genes were expressed in bacteroids in the nodule of *A. sinicus* (Sriprang et al. 2003; Ike et al. 2007) infected with the recombinant strain B3. They have also evaluated the amount of Cd accumulated in the nodule as a result of the expression of these two genes. However, the increase in the amount of Cd accumulated in the nodule was not sufficient compared with that in free-living culture (Ike et al. 2007). Introduction of AtIRT1, an iron-regulated transporter 1 (IRT1) that takes up Fe(II) and the first member of the zinc-regulated transporter, in the recombinant strain B3 which advantaged the accumulation of Cu and As in the nodules of *A. sinicus* (Ike et al. 2008). The Cd accumulation in the free-living

rhizobial cells containing AtPCS and AtIRT1 showed significant enhancement compared with that in the cells containing only AtPCS, suggesting that the presence of the AtIRT1 transporter protein enhances the transport of Cd to recombinant B3 free-living cells carrying AtPCS and accelerates the Cd accumulation.

Conclusion

The use of plants and microorganisms to extract, sequester, or detoxify pollutants is generally called as bioremediation. Bioremediation offers a low-cost method for soil or water remediation, and some extracted metals may be recycled for value. It has been found that legume–rhizobia symbiosis can be utilized as a model system to achieve two goals: (1) heavy metal remediation and (2) improving nitrogen pool through symbiosis. However, scientific understanding is needed to harness the natural processes and to develop methods for accelerating these processes for the bioremediation of contaminated environments. Even though rapid progress has been made in the last few years, more is required to popularize and adopt this strategy in the metal cleanup program. The use of culture-independent molecular techniques has definitely provided some clues to better understand the microbial community dynamics and structure and has assisted in providing the insight into the finer details of bioremediation which has surely facilitated to make the technology safer and reliable. In this context, *Rhizobium*–legume symbiosis can be exploited for rehabilitation of heavy-metal-contaminated environments—an emerging area of interest because it provides an ecologically sound and safe method for restoration and remediation. The application of transgenic plants on the contrary in phytoremediation of contaminated environments provides many advantages over the use of simple hyperaccumulating plants. For example, it has now become feasible to increase a plant's capacity to tolerate, accumulate, and/or metabolize pollutants leading to the production of large biomass. With the exciting new developments taking place around the world in this field and considerable focus on interdisciplinary research while using the current technologies as genetic manipulation for rhizobia, it is likely that the current problem of heavy metal toxicity could at least be sorted out. This technology is expected to be used on a wider scale for cleaning the environment in near future. Considering these, it can be suggested that legume–*Rhizobium* symbioses could play important roles in making the environment free of contaminants vis-à-vis maintaining soil fertility.

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Microbially Mediated Transformations of Heavy Metals in Rhizosphere

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Abstract

A complex interaction occurring between various plants and rhizosphere microorganisms governs the physicochemical and biotic characteristics of soils. And hence, the composition and functional properties of agronomic soils are often significantly different from those of bulk soil. Presence of heavy metals in soil resulting from the natural processes or introduced through anthropogenic activities affects growth and activity of plants and microbes. Therefore, the selection of microbial strains resistant to heavy metals and plants capable of accumulating excessive concentration of metals often called hyperaccumulating plants becomes important in remediation technologies. Beneficial soil micro-organisms, both free living and symbionts, can stimulate plant growth, ease toxicity, and enhance accumulation of metals in plants. Amendment of soil properties by enrichment with organic matter (biosolid, compost) and cultivation of plant species inoculated with metal-resistant microbes are likely to improve efficiency of phytoremediation and reduce environmental risks associated with heavy metal contamination. This chapter presents plant–microbe interactions and the mechanisms involved in the mobilization, transfer, and stabilization of metals in soil by rhizosphere microbiota.

8.1 Introduction

Industrial activities and agricultural practices introduce large amounts of heavy metals into the environment. There is evidence that the concentration of heavy metals (HM) in food and fodder correlates with those in the upper layers of

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cultivated soils (Van der Perk 2006). In soil, the aqueous phase generally contains metals in ionic form and as organic and inorganic complexes. In addition, aqueous phase is a source of water and inorganic nutrients for plants as well as provides organic substrates to microorganisms. However, the pool of metals in liquid phase remains in equilibrium with those metals in the solid phase and is the net result of sorption/desorption processes occurring between different compounds of soil–solid phase (Gupta et al. 1988). Adsorption governs HM distribution between the solid phase of soil and soil solution. Desorption, on the other hand, relieves HM from the solid phase into soil solution. Both processes occur simultaneously in soil and control mobility and bioavailability of metals (Christensen and Huang 1999).

The major processes that increase the retention of HM in soils are (1) adsorption: metal binds to the negatively charged exchange site of clay minerals, organic matter, and soil microorganisms; (2) formation of complexes with organic matter; (3) precipitation as carbonates, phosphates, sulfides, and hydroxides; and (4) coprecipitation with calcite and iron (Fe), aluminum (Al), and manganese (Mn) oxyhydroxides. Retention of metal immobilized by various soil constituents is, however, affected by different physicochemical variables of aqueous phase (Ernst 1996; Majewska and Kurek 2007). All these processes in general are controlled by variable metabolic activities of soil microorganisms and plants and also by abiotic factors like, pH, redox potential, etc. These factors together determine the amount of bioavailable metals in soils. Therefore, analysis of the aqueous phase could be an important parameter to understand the chemical or biological changes in soil ecosystem. However, it does not provide any information regarding the contribution of individual chemical, biological, and physical processes (Gupta and Aten 1993). There are reports that under laboratory conditions ternary systems, such as humic acids–bacteria–metal (Wightman and Fein 2001), clay–fungal biomass–metal (Morley and Gadd 1995), or clay–bacterial envelopes–metal (McLean et al. 2002), immobilize quite different amounts of metal than would be expected after the summation of the amounts of metal sorbed by their individual components under the same conditions. These findings indicate that, in soil, interactions between biotic and abiotic solid phase constituents have a significant influence on sorption/desorption processes of metals. The biggest number and highest activity of microorganism in soil occur in rhizosphere enriched with root exudates. So, their effect on heavy metals bioavailability can be robust (Huang and Germina 2002).

8.2   Plant Growth Effect on Microbial Rhizosphere Population

Plants are the main source of organic matter accounting for about 1–8% of the weight of most soils. It is the result of decomposition of aerial plant part but mostly originate from root. Generally, 30–60% of photosynthates generated in annual plants are deposited into roots, and 4–70% of C is lost into the rhizosphere within hour as rhizodeposits. Major components of rhizodeposition are soluble

low-molecular-weight (LMW) organic compounds like sugars, organic acids, amino acids, phenolics, vitamins, and siderophores. These substances are present in the cytoplasm, and their concentration varies greatly in proportions with plant species and cultivars. Mucilage, consisting mainly of polysaccharides such as polygalacturonic acids and extracellular enzymes, and insoluble root border cells (RBC) and sloughed dead cells are the principal components of rhizodeposition. The amount of organic compounds into rhizosphere is influenced by plant age and various biotic and abiotic stresses. The rhizosphere environment is different compared to bulk soil with respect to physical, chemical, and biological properties (Kumar et al. 2006). For example, the changes in rhizosphere pH are often higher by one unit and sometimes by more than two units compared to bulk soil. The rate of pH changes in rhizosphere is affected by both plant and soil factors (Marschner and Römheld 1996). Excretion or reabsorption of H^+ or HCO_3^-, evolution of CO_2 by root respiration, and organic acids contained in root exudates released into rhizosphere are other important plant factors. The form of N used by plants (NO_3^-, NH_4^+, N_2 fixation) has prominent influence on rhizosphere pH (Thomson et al. 1993). The bulk as well as rhizosphere soils are heterogenic with respect to redox potential. Even in aerial soils, anaerobic microsites are found which are more common in the rhizosphere than in bulk soil due to O_2 consumption by roots and microbial respiration. However, in wetland, O_2 transport from shoot through parenchyma to roots and its release into rhizosphere allow to maintain high redox potential (Huang and Germina 2002).

Rhizodeposits are the major source of substrates for microbial growth and activity in the rhizosphere. They are also involved in: (1) chemotaxis acting as attractants (e.g., flavonoids, aromatic amino acids, dicarboxylic acids) or repellents; (2) hormonal activity; (3) chemical defense against competitive plant species and deleterious microorganisms; and (4) they may have enzymatic properties (Kumar et al. 2006). The population number and dynamics in rhizosphere are significantly greater than in soil not influenced by roots. The ratio of microbial number in rhizosphere (R) to the number of microbes in bulk soil (S) called rhizosphere effect for growing plant is usually higher for bacteria (including actinomyces) than for fungi and ranges for culturable bacteria from 2 to 20. Typically, bacterial rhizosphere population number ranges from 10^6 to 10^9 cells g^{-1} soil. Number of fungi in rhizosphere can be 10^3–10^5 propagules g^{-1} soil, and R/S values range from 3 to over 200, but usually remain between 10 and 20 (Huang and Germina 2002). The enhanced microbial population in rhizosphere may be either beneficial or detrimental to plant growth. Beneficial effects of microbes are mobilization of nutrients from soil or nutrients supply to plants and production of phytohormones. Detrimental effect may include competition for substrates and plant pathogenesis (Khan 2005; Khan et al. 2009).

Microorganism can produce and sense signal molecules allowing the whole population to develop biofilm over the root surface and initiating a concerted action when a particular population density is achieved. These phenomena are known as quorum sensing (Daniels et al. 2004). Bacteria monitor their own population density by production of the low-molecular-weight signal molecules called autoinducer or quormon. Their extracellular concentration is proportional to the

population density of producing organism. Different signal molecules to measure population density are used by Gram-positive and Gram-negative bacteria. One of the known mechanisms by which bacteria can communicate with each other is production and release of signal molecule *N*-acyl-homoserine lactone (AHL) into the environment (Fuqua et al. 1996). Different species of bacteria can produce the same or very similar signal molecules which make possible interspecies communications (Smith and Ahmer 2003). Six bacterial strains able to degrade AHL were isolated from tobacco (*Nicotiana tabacum* cv. Samson) rhizosphere, suggesting that microbial consortia are involved in quorum-sensing signal turnover (Uroz et al. 2003).

Microorganisms influenced by roots are categorized as infecting the plant (living inside the roots) and as noninfecting (living in very close proximity to root surface and attached to the root surface). Soil microorganisms including free living and symbiotic bacteria and fungi are considered as integral part of rhizosphere biotic community (Van der Lelie 1998). Plant growth-promoting rhizobacteria (PGPR) capable of aggressively colonizing plant roots are broadly divided into free living and symbiotic groups. These organisms can affect plant growth in three different ways: (1) by synthesizing and providing particular compounds to the plants, (2) by facilitating the uptake of certain nutrients from environment, and (3) by protecting plants from certain diseases. Increased plant growth is due to the availability of phytohormones, vitamins, enzymes, siderophores, antibiotics, and 1-aminoacy-lopropane-1-carboxylate (ACC) deaminase synthesized by PGPR. Other properties important for promoting plant growth include the ability of PGPR to solubilize inorganic P, mineralization of organic P, improving plant stress tolerance to drought, salinity, and metal toxicity. Ethylene is phytohormone involved in various processes including leaf senescence and abscission, fruit ripening, regulation of rhizobial Nod factor signaling, and nodule formation and is a significant part of plant defense system. At higher concentration, ethylene, however, inhibits plant growth and development. Ethylene production, on the other hand, can be stimulated by plant infection caused by rhizobacteria. PGPR can synthesize ACC deaminase, which transform the ACC (precursor of ethylene synthesis) to ketobutyrate and ammonia and thereby decrease the plant stress caused by higher ethylene concentration (Wenzel 2009).

Roots of approximately 80–90% of land plants growing in natural agricultural ecosystems are colonized by arbuscular mycorrhizal fungi (AMF). In forest soil, the most common mycorrhizal association is the ectomycorrhizal (EM). AM fungi recognize their host by signal compounds released by host roots and thereafter establish a functional symbiosis. Host root exudates stimulate spore germination and early growth of AMF hyphae. For example, glycosylflavonoids isolated from nonmycorrhizal roots of melon (*Cucumis melo*) plant when applied to AMF-inoculated melon plants were found to enhance root colonization (Akiyama et al. 2002). However, both AM and EM are involved in uptake and increasing the availability for plant nutrients such as P, NH_4^+, NO_3^-, K, Ca, SO_4^-, Cu, Zn, and Fe. Mycorrhizal associations also affect plant growth indirectly by increasing

the stability of soil aggregates, modifying rhizosphere pH and Eh, and suppressing soil-borne fungal pathogens (Khan 2005).

The secondary metabolites released by roots play an important role in establishing specific and intense associations between plant and rhizospheric microbes. These interactions that could range from a positive mutualistic interaction to negative pathogenic association can in turn affect plant growth and health severely. Root exudation is the mechanism that allows a plant to regulate the composition of rhizosphere microbial community. Broeckling et al. (2008), for instance, using real-time PCR analysis of fungal community in rhizosphere of two plant species, namely, *Arabidopsis thaliana* and *Medicago truncatula*, found that both plants were able to maintain resident soil fungal population but were unable to do it with nonresident population. Resident treatment included *A. thaliana* grown in Illinois soil and *M. truncatula* grown in Texas soil. Nonresident treatment included *M. truncatula* in Illinois soil *A. thaliana* in Texas soil or either plant species grown in Oregon soil. A net increase in fungal biomass was found when nonresident root exudates were added to resident plant treatment. These findings indicate that root exudates are not only part of lost plant C into rhizosphere but can also modify the composition of soil fungi. Root exudates through its antifungal effect can thus reduce the relative abundance of fungal species or may positively regulate the relative abundance either through growth-inducing chemical signals or by supplying carbon substrates.

8.3 Mobilization of Bioavailable Pool of Heavy Metals in Rhizosphere

Environmental bioavailability is defined as the amount of chemical (element) present in soil in forms that biota (e.g., plant) can take up during the time they are growing (Chaignon et al. 2009). Proportion between amounts of HM present in solid soil fractions and soil solution determines the amount of bioavailable HM. However, the pool of soluble metals in soil usually represents a very small fraction of total amount of HM (He et al. 2005). The concentration of soluble HM in soil solution decreased by plant or taken up by other biota is replaced by their desorption from surface of solid soil constituents. Desorption, an important process affecting concentration of HM in soil solution, is strongly dependent on soil pH. Percentages of Cd, for example, desorbed were 80–90% in Chinese ultisol (pH_{KCl} 3.81) and 25–28% in oxisol (pH_{KCl} 5.4) (Wang et al. 2009). Similarly, the desorption efficiency was greater than 80% for Ni, Cu, Zn, and Cd on ferrihydrite at pH 4.5 (Schultz et al. 1987), while Cd release rate was very low at pH 5 but increased exponentially as pH decreased below 4.5 for cultivated soil (Strobel et al. 2001). The form of N applied to plants can also significantly influence the soil pH. As an example, Bravin et al. (2009) observed that soil pH was significantly increased to 6.9–7.6 when durum wheat (*Triticum turgidum durum* L.) was nitrate fed. Change in N (e.g., NH_4NO_3) acidified (pH 3.9) the soil. It was found that Cu bioavailability (measured as Cu concentration in plant) was 2.4- to 4.2-fold higher for NH_4NO_3-

fed plant and was increasing with decreasing pH. Other important soil factor regulating pool of bioavailable HM is organic matter, mainly dissolved organic carbon (DOC). Dissolved organic matter (DOM) can mobilize HM by forming a soluble stable complex, while solid organic matter immobilizes HM (Zhao et al. 2007b). However, direct activity of plants and their indirect effect also transform insoluble OM into DOC (Yang et al. 2010). Even though the source of DOC can be root exudates, it may also be formed by the microbial decomposition of older insoluble soil OM (Zhao et al. 2007a). For example, a measurable increase in Pb–fulvic complexes, Pb–humic complexes, organic Pb, and amorphous Pb was recorded in rhizosphere of *Elsholtzia splendens* and bulk soil. The accumulation of OM in soil was suggested due to the root exudation and the enhanced microbial activity in the rhizosphere. Furthermore, the regression analysis indicated that Cu mobilization was increased by soil-borne DOC, but Zn and DOC were poorly correlated compared to Cu and DOC. The vertical changes in DOC and metal concentrations within the top soil, however, suggested a faster mobilization of metals at the soil surface. This was attributed due to decomposition and mineralization of soil OM caused by microbes, desorption of metals from mineral surfaces, and a slower mineralization rate in deeper layers. For example, plants grown in pot soil environment increased the mobilization of HM from soil. The results of column and field lysimeter experiments, however, indicated that plants can cause both immobilization and mobilization of HM which depend on plant species, tested metal species, and soil characteristics (Track et al. 1998; Zhu et al. 1999). On the other hand, Zhao et al. (2007b) reported that leaching of metals like Cu and Zn from soil column was much slower in the presence of plants compared to those grown in the absence of plants. The metal uptake by *Salix viminalis* was two times more than the leaching from top soil to subsoil for Cu and about 30 times greater for Zn. While comparing the concentration of metals mobilized in the presence and absence of plant, it was shown that DOC was mobilized by microbial activity from soil OM but not root exudates that controlled the mobilization of Cu and Zn. Carboxyl and hydroxyl functional groups of humic acid (HA) and fulvic acid (FA) can form very stable complexes with metal cations or hydroxyl metal cations. Considering these facts, Wang and Mulligan (2009) confirmed that HA could enhance mobilization of Zn, Pb, Cu, and As from the mine tailings under alkaline conditions. The mobilization of these elements was found to be positively correlated with the mobilization of iron. Further amendment of top layer of landfill covering soil with municipal waste compost resulted in significant increased metal (Cu, Zn, Pb) contents in top soil.

LMW organic acids (OA) such as citrate and oxalate, exuded by plant roots or produced by microbial activities, are also involved in mobilization or translocation of metals in soil. They can form soluble complexes with metals and also acidify root zones. As an example, Naidu and Harter (1998) reported that amounts of metals extracted from soil by a mixture of OAs were well correlated with amounts of mobile metal fraction present in soil solution. In other study, Labanowski et al. (2008) found two metal pools as readily labile and less labile in soil contaminated with metallurgical fallout. In citrate extracts, sum of both pools of Zn and Cd was proportionally higher than Pb and Cu and proportions of Pb and Cu extracted with

EDTA were three times higher than when extractant was citrate. Proportions of citrate extracted (labile) metals were found constant with their short-time in situ mobility assessed in the studied soil.

8.4   Effects of HM on Soil Microorganisms

Soil microorganisms are the first organisms affected by a HM-contaminated environment (He et al. 2005). The effect of HM on a microbial community depends on the source, form, concentration, and properties of both the individual HM and soil as well as the time of exposure of biota. Among metals, some like Zn, Ni, and Cu are considered to be essential "trace elements" with important roles in biochemical reactions; however, at higher concentrations, all metals become toxic (Kabata-Pendias and Pendias 2001). In order to cause damage, a heavy metal must enter the cell. Once metals are inside, heavy metal cations may inhibit the activity of sensitive enzymes. Other toxic mechanisms include (1) interactions of heavy metal ions with physiological ions, for example, Cd^{2+} with Zn^{2+}, Cd^{2+} with Ca^{2+}, Ni^{2+} with Fe^{2+}, and Zn^{2+} with Mg^{2+}, which abolish the function of the latter; (2) binding of heavy metal cations to glutathione in Gram-negative bacteria, which results in the formation of bisglutathionate complexes able to react with molecular oxygen to give products such as oxidized bisglutathione (GSSG), metal cations, and H_2O_2; and (3) interference of heavy metal oxyanions, such as chromate, with the metabolism of structurally related nonmetals (e.g., sulfate) (Nies 1999).

It is well known that the presence of HM in soil at higher concentrations affects the growth, activity, and community structure of soil microorganisms. A soil microbial community may react to higher concentrations of HM with a decrease in biomass as a result of direct killing or biochemical disability of organisms. Long-term exposure to lower HM concentrations has shown a decreased metabolic efficiency in microbes. In a soil contaminated for a long time with HM ($31–1,845$ mg Pb kg^{-1}, $27–162$ mg Cu kg^{-1}, and $81–4,218$ mg Zn kg^{-1}), a significant negative correlation was found among microbial biomass, soil OC, total N and C mineralization, and HM contents. However, the specific respiration rate and nitrification were not affected by HM contents (Vásquez-Murrieta et al. 2006). Addition of HM contained in a biosolid to a neutral loaming soil caused a roughly 36% reduction in C mineralization, a decrease in microbial biomass C, a reduction in N mineralization and an average 40% decline in microbial respiration compared to a mixture of soil and a biosolid noncontaminated with HM. The decrease in C mineralization was probably caused by complexation of part of Cu, Pb, and Zn added with the biosolid with organic matter, which prevented decomposition by microorganisms of C in the complexes (Kao et al. 2006).

Correlation analysis demonstrated that toxicity of heavy metals to soil microorganisms was affected by their total and water-extracted concentrations (Płaza et al. 2010). Correlation analysis among basal respiration, biomass C, utilization of sole C sources, and heavy metal fractions from sequential extraction indicated that respiration was negatively correlated with soil Cd, Cu, Ni, and Zn

concentrations, whereas microbial biomass was negatively correlated with Pb concentration in soil. Concentration of Ni in the exchangeable fraction and the iron- and manganese-oxide-bound fraction was the most important factor for the shift in community-level physiological profiles of soil microbial communities assessed by the sole C source utilization test (Yuangen et al. 2006). The toxic effect of HM on a soil microbial community can be measured as a change in heat production by microorganisms resulting from metabolic processes occurring in living cells. Yao et al. (2008), for example, using microcalorimetric method, found that increase in the amount of hexavalent chromium in soil decreased soil microbial activity, probably due to a strong toxic effect on the microbial life.

8.5   Effect of Microbial Activity on Metal Toxicity to Plants

Improving plant–microbes interactions and inoculating plants with PGPR can be beneficial for increasing biomass of plants grown in soils even contaminated with pollutants (Singh et al. 2010). The role of PGPR in development of plants especially in HM-contaminated soils can be even more important because of their involvement in increasing the plant tolerance to heavy metals. The rhizosphere microorganisms have the ability to reduce the toxicity of heavy metals. The microorganisms achieved this by effluxing metal ions outside the cell, reducing toxic form of metal to less toxic forms and by accumulating and complexing metal ions inside the cell (Wani et al. 2007, 2008b).

Chromium(III) is an essential micronutrient in human diet and is relatively less toxic and less soluble than Cr(VI), which is more toxic, carcinogenic, mutagenic, and more easily available for uptake by plant roots (Faisal and Hasnain 2005). However, inoculation of plants (*Vigna radiata* var. NM-92) with PGP chromium-tolerant bacterial strains (*Bacillus cereus* S-6 or *Ochrobacterium intermedium* CrT-1) significantly increased the plant biomass compared to noninoculated plants. When *Vigna radiata* var. NM-92 was grown in soil treated with 300 μg g^{-1} of hexavalent chromium, the bioavailability of Cr was increased at acidic pH. Plants inoculated with chromium-resistant bacterial strains *Ochrobacterium intermedium* (isolate CrT-1) or *Bacillus cereus* (isolate S-6) resulted in significant plant growth stimulation under Cr stress and decreased chromate contents in the plants. The bacterial strains also reduced Cr(VI) to Cr(III) (Faisal and Hasnain 2006). In other similar study, *Ochrobacterium intermedium* inoculation increased the plant (*Helianthus annuus* var. SF-187) weight by 20% and auxin content by 69%, while it decreased chromium uptake by 30% in sunflower grown in soils treated with 300 μg g^{-1} of Cr(VI) (Faisal and Hasnain 2005). Chromium-tolerant consortia of four rhizobacterial strains isolated from rhizosphere of *Vigna radiata* grown in soil amended with tannery sludge increased significantly the root length (138%), shoot length (88%), biomass (25%), and total chlorophyll compared to non-inoculated green gram plants. A similar increase in the overall growth of *Mesorhizobium* strain RC3 inoculated chickpea (*Cicer arietinum* L.), grown in the presence of hexavalent chromium, is reported by Wani et al. (2008a).

Heavy metal-tolerant rhizosphere microbial community can alleviate toxicity and enhance plant growth by synthesizing phytohormones. Moreover, plant reacts to various environmental stresses such as a high concentration of heavy metals by synthesizing "stress" ethylene. Microbes able to produce ACC deaminase can transform 1-aminoacylpropane-1-carboxylate (precursor of ethylene synthesis) to ketobutyrate and ammonia (Khan et al. 2009). For example, PGP heavy metal-tolerant bacterial strains identified as *Variovorax paradoxus*, *Rhodococcus* sp., and *Flavobacterium* sp., isolated from highly Cd-contaminated sewage sludge, mining waste, and root zone of *Brassica juncea* L. Czern seedlings grown in Cd-supplemented soil have been shown to produce IAA, siderophores, and ACC deaminase. Inoculation of *B. juncea* seedlings with these strains resulted in root elongation, when grown either in the absence or presence of Cd. A positive correlation between in vitro activity of bacterial ACC deaminase and their stimulatory effect was found for root elongation. Strain *V. paradoxus* could transform ACC and also utilized this compound as sole C and N source (Belimov et al. 2005). Inoculation of canola seeds (*Brassica campestris* cv. Tobin) with plant growth-promoting and metal-resistant strain SUD165 of *Kluyvera ascorbata* protected seedlings against Ni toxicity and prevented its accumulation in roots and shoots (Burd et al. 1998). In a similar study, bacteria belonging to genera *Bacillus*, *Micrococcus*, *Arthrobacter*, *Pseudomonas*, *Sphingomonas*, *Microbacterium*, *Acinetobacter*, *Rahnella,* and *Azotobacter* were isolated from copper-tolerant plants rhizosphere, growing in a copper mine wasteland. Some of these bacterial strains produced IAA, siderophores, and ACC deaminase and could solubilize insoluble phosphate. Following inoculation, an increase of 16–41% in root elongation of rape plants (*Brassica napus* var. Qinyou-7) was observed (He et al. 2010). Ghorbanli et al. (1999) indicated that change in the concentration of other phytohormone, gibberellin, can also affect toxicity of Cd to the plant (*Glycine max* L. cv. Pershing). Addition of this compound (10 mg m^{-3}) to Hoagland nutrient solution supplemented with Cd^{2+} caused a partial removal of Cd effects such as reduction of root and shoots dry matter production and increased leaf area and stem length.

Studies of the effect of legumes inoculated with PGPR and HM-tolerant rhizobia have revealed that metal-tolerant rhizobia can affect the performance of legumes when grown in metal-stressed soil. Wani et al. (2008b) found that inoculation of pea (*Pisum sativum*) grown in metal-amended soil (580 mg Ni kg^{-1}, 9,580 mg Zn kg^{-1}) with metal-tolerant PGP *Rhizobium* sp. RP5 increased plant biomass by 46–65% in the presence of Ni and by 47–54% when Zn was added, and reduced the accumulation of both the metals in plant organs. Inoculation of green gram plants (*Vigna radiata* L.) with PGP *Bradyrhizobium* sp. (*vigna*) RM8 tolerant to Ni and Zn enhanced nodulation, nodule contents of leghemoglobin, seed yield, and grain protein when the plant was grown in a soil containing 290 mg Ni kg^{-1} and 4,890 mg Zn kg^{-1}. The uptake of the metals into plant organs was also reduced in inoculated plants. The authors suggest that the increased growth of plants in the presence of *Bradyrhizobium* sp. (*vigna*) was possibly due to the effect of bacterial metabolites such as IAA, siderophores, and ammonia (Wani et al. 2007).

8.6   Phytoremediation of Metal-Contaminated Soil

One of the methods of bioremediation of a contaminated environment is phytoremediation. Of the various phytoremediation strategies, phytoextraction is used to remove contaminants from soil, sediments, or water by plant uptake. For successful phytoextraction, it is necessary to clean the site to a level that satisfies environmental regulations. Cleaning of a contaminated medium using phytoremediation is inexpensive compared to other conventional technologies employed for cleaning up polluted sites (Nevel et al. 2007; Robinson et al. 2006). Unfortunately, this technique shows low efficiency and is time-consuming. It takes a hyperaccumulator plant (such as *Thlaspi caerulescens* or *Salix* sp.) more than 10 years to slightly decrease the total Cd concentration in the upper 0.5 m of soil by accumulating this element in its tissues. For this reason, sometimes bioavailable contaminant stripping, a technique which extracts only the most labile bioavailable pools of hazardous metals, is used for cleaning of contaminated sites (Nevel et al. 2007).

Plants used for phytoextraction have been categorized as metal hyperaccumulators. Hyperaccumulator species of plants are those whose leaves contain more than 10,000 mg Zn and Mn kg^{-1}, 1,000 mg of Ni and Cu kg^{-1}, and 100 mg of Cd kg^{-1} dry weight when grown in metal-rich soil (Abou-Shanab et al. 2006, 2010; He et al. 2005). The majority of the hyperaccumulator plant species studied so far are Ni hyperaccumulators, characterized by small size and slow growth, such as *Alyssum murale*, *Arabidopsis halleri*, and *T. caerulescens* (Abou-Shanab et al. 2003; Farinati et al. 2009). Hyperaccumulators of Cd, Pb, Zn, Co, Cu, and As are relatively less investigated (Nevel et al. 2007). To explain the rapid and efficient uptake of Cd and Zn by ecotypes of the hyperaccumulator *T. caerulescens*, three mechanisms related to the key processes of root proliferation and effective uptake have been proposed: (1) a higher density of Zn and Cd transporters in root cells, (2) an active proliferation of root branches toward Zn-/Cd-rich patches supported by a large cumulative root density to above-ground biomass ratio plus a larger proportion of fine roots compared to other plants, and (3) enhanced root to shoot translocation, which reduces metal accumulation in roots (Dessureault-Rompré et al. 2010). High constitutive expression of genes involved in metal uptake, transport, and cellular detoxification was found in *A. halleri*, a hyperaccumulator of Zn and Cd (Farinati et al. 2009). Studies by Lombi et al. (2000) indicated that the mechanisms of Cd and Zn hyperaccumulation were not identical in *T. caerulescens*. Instead, specific Cd transporters, which differed from those responsible for Zn uptake, were found. However, it was observed that root exudates of *T. caerulescens* did not contain significantly more chelating components with a high affinity for metals than root exudates of nonaccumulators, suggesting that hyperaccumulators are not more efficient in mobilizing metals from nonlabile pools in soil (Zhao et al. 2001). The significantly higher content of carbonate-bound Cd in the rhizosphere of Cd accumulators (e.g., *Brassica napus*) than in the rhizosphere of

nonaccumulator species (Su et al. 2009) indicates an indirect effect of rhizosphere microorganisms on the bioavailability and uptake of HM by hyperaccumulators (Abou-Shanab et al. 2003). Microorganisms can affect heavy metal mobility and availability to the plant due to release of chelators, acidification, and changes in the redox potential (Abou-Shanab et al. 2006). Introduction of *Microbacterium oxydans* AY509223 into the root zone of *A. murale*, a Ni hyperaccumulator, significantly increased (on average by 30%) Ni uptake when the plant grew in Ni-rich soil (Abou-Shanab et al. 2006). Further results of studies on the rhizosphere of Ni, Zn, and Cd hyperaccumulators (e.g., *Thlaspi caerulescens*) suggest that mobile and labile metal–DOM complexes play a key role in the replenishment of available metal pools in the rhizosphere of hyperaccumulating ecotypes (Dessureault-Rompré et al. 2010). Increased proportions of Ni-resistant, acid-producing bacteria (20%) were found in the rhizosphere of the Ni hyperaccumulator *A. murale* grown in Ni-rich soil compared to bulk soil (<6%). Fresh and dry weight and Ni and Fe concentrations were higher in shoots of the hyperaccumulator *A. murale* growing in contaminated nonsterilized soil than in those growing in sterilized soil.

A second type of plants used with success in phytoextraction are metal-tolerant species which can take up large quantities of metal contaminants from soil due to their high biomass but modest metal accumulating ability of their tissues (Clemens et al. 2002; Yang et al. 2002). A native Chinese herb, for example, *Elsholtzia splendens*, accumulated 2,288 mg Cu kg^{-1} in roots and 304 mg Cu kg^{-1} in shoots, producing, at the same time, a large biomass (11,000 kg ha^{-1} of shoots) (Jiang et al. 2002, 2004). Trees can be an alternative to small-biomass hyperaccumulators, due to their large root systems, high transpiration rates, rapid growth, and large biomass production (Pulford and Watson 2003). After 3-year growth, large concentrations of Cd (250 mg kg^{-1}) and Zn (3,300 mg kg^{-1}) were determined in leaves than roots of *Salix* × *smithiana* grown on a soil containing 13.4 mg kg^{-1} Cd and 955 mg kg^{-1} Zn. The values of bioaccumulation factors were 27 for Cd and 3 for Zn. The total concentrations of Cd and Zn in soil after 3 years' growth of this tree were reduced by 20% and 5%, respectively (Wieshammer et al. 2007).

Phytostabilization, another phytoremediation technique, is used to minimize metal mobility in soils containing high HM concentrations (Ernst 2005). Trees, with their deep extensive rooting, serve as good candidates in phytostabilization method. However, ability to acidify root zone and translocate the metal to their leaves are the two characteristics which determine the outcome of phytostabilization process. Mertens et al. (2007) reported that the concentrations of Cd and Zn in the upper soil layer under a 33-year-old poplar (*Populus* "Robusta") were 2–3 times higher than under oaks (*Quercus robur*) of the same age. Part of the metals taken up from the entire rooting zone was translocated to the above-ground plant parts and return into top soil with falling leaves. Their decomposition by microbial activity supplies organic–metal complexes, and metals associated with organic matter are more mobile and bioavailable compared to metals adsorbed on mineral particles.

8.7   Immobilization of HM in the Rhizosphere

Heavy metals can be immobilized in soil by sorption, coprecipitation, and ion substitution or through redox changes. These processes can be enhanced by direct or indirect microbial activity. In the environment, there can be found local sites with natural accumulation of heavy metals called geochemical barriers. In these sites, the conditions for HM migration, mobilization, and accumulation alter distinctly over time under the influence of microbial activity (Burkhardt et al. 2009). Microbial reduction of sulfates results in precipitation of metal sulfides, and microbial biomass acts as a sorbent of metal compounds. Inaba and Takenaka (2005) studied microbial contribution to the fractionation of Cu in a forest soil. Dramatic changes in Cu fractionations such as an increase in the carbonate fraction of Cu accompanied by a decrease in its residual fraction were found after 3 months. However, the Cu fraction referred to by the authors as "residual fraction" did not represent Cu in crystal structures of soil minerals, as defined by Tessier, but the metal in soil organic–clay mineral complex. The results suggested that Cu partitioned in the persistent organo-bound fraction could be degraded into carbonate fraction by microbial activity.

Besides the effect of microbial metabolism on the mobility of HM in soil, microorganisms can immobilize HM by acting as efficient biosorbents. For instance, the bacterial strains *Ralstonia* sp. and *Bacillus* sp. isolated from Korean soil contaminated with diesel oil and HM were used by Choi et al. (2009) as biosorbing materials to remove HM from aqueous solution. Of these, biomass of *Bacillus* sp. was found more efficient than *Ralstonia*. Biosorption was pH dependent and reached to a maximum level at pH 3–5, occurring mostly at the bacterial cell wall (Choi et al. 2009). Similarly, the fungal biomass of *Trichoderma koningii* very efficiently sorbed the Cd (Kurek and Majewska 2004) and immobilized seven times more Cd than the same amount of clay (montmorillonite), five times more than humic acids (commercial product), and 300% of the amount of Cd immobilized by the soil bacteria *Arthrobacter* spp. L II (Fig. 8.1). The immobilization of Cd by fungal biomass was much more robust in comparison to immobilization by other soil constituents. During extraction of Cd with 0.1 M $NaNO_3$ solution (pH 6), only 5% of immobilized Cd was released from *T. koningii* mycelium whereas 50% of the metal was released from montmorillonite and 30% each from humic acids and the biomass of *Arthrobacter* spp. L II. Solution of 0.1 M $NaNO_3$ is recommended by the Swiss Federal Agricultural Chemistry and Environmental Hygiene Institute, which allows a good estimation of metal available for plant uptake by simulating the ionic strength of the soil solution (Keller and Védy 1994). These and other findings (Choi et al. 2009; Kurek and Majewska 1998, 2004) indicate that the direct effect of microbes including bacteria and fungi on the amount of phytoavailable toxic metals in the root zone cannot be ignored (Fig. 8.2). Furthermore, transformation of plant and animal residues into soil OM like humus and its derivatives is an important indirect result of microbial activity. Application of chicken manure compost to soil treated with various levels of Cd (0–50 mg kg^{-1}) resulted in an over 70% decrease in the exchangeable/soluble Cd
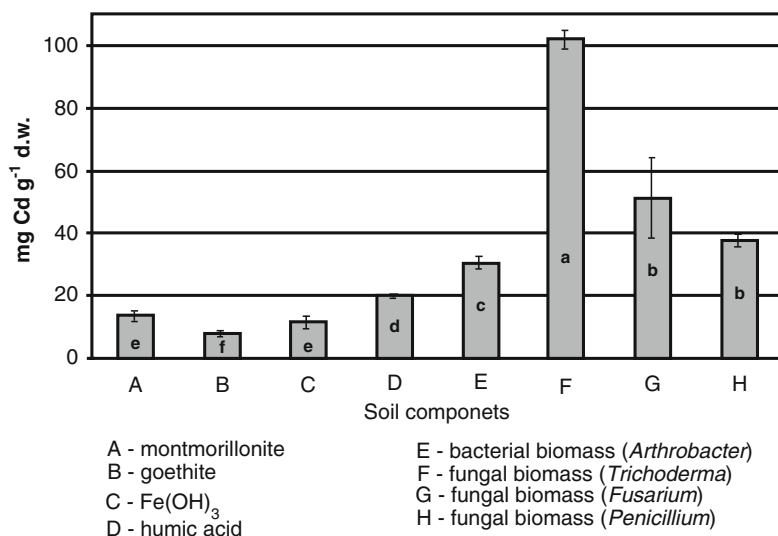
Fig. 8.1 *In vitro* immobilization of Cd from water solution by soil constituents after 12 h under the same conditions (500 μg Cd ml^{-1} and 2.5 g d.w. mass l^{-1}, pH 6, 5). *Bars* marked with the same letters are not significantly different ($p > 0.05$). Standard deviations are shown as deviation bars ($n = 5$)
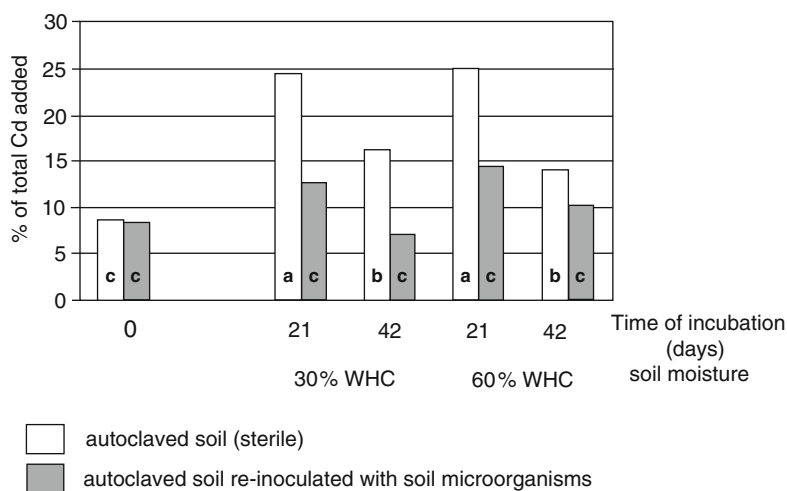
Fig. 8.2 Effect of soil microbes on changes of soluble plus exchangeable fractions of Cd (extracted from soil with 0.1 M NaNO$_3$) in soil supplemented with 10 mg Cd kg^{-1} (as water solution of CdCl$_2$). *Bars* marked with the same letters are not significantly different ($p > 0.05$). *WHC* water-holding capacity

fraction, but increased the concentration of organic-bound and inorganic precipitate forms in soil. Bioavailability of metals assessed as Cd uptake by wheat (*Triticum aestivum*) was also reduced by 50% as an effect of increased soil pH, complexation of Cd by OM, and coprecipitation with P (phosphate) added with compost (Liu et al. 2009).

Conclusion

Cleaning of HM-contaminated soil is usually aimed at decreasing the concentration of HM by their transfer with biological or chemical methods to other environmental compartments. Phytoremediation is a method which integrates the processes occurring in the three-component ecosystems (soil/plants/microorganisms). The efficiency of phytoremediation techniques, for example, those of phytoextraction and phytostabilization, depends on the size of the bioavailable pool of heavy metals in soil and the ability of plants to consume these elements as well as the size of plant biomass produced. Inoculation of plants with beneficial microorganisms, including free living and symbiotic associates, is likely to enhance dry matter accumulation in plant (biomass) and, concomitantly, the accumulation of HM. This in turn may alleviate the toxicity of deleterious elements from contaminated soil ecosystems. However, concerted efforts are required to find microbes and plant genotypes better equipped with metal reducing/detoxifying ability so that upon inoculation they could be used in heavily polluted soils.

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Rhizoremediation: A Pragmatic Approach for Remediation of Heavy Metal-Contaminated Soil

9

Velmurugan Ganesan

Abstract

Soil pollution is the primary source that transmits pollutants like heavy metals from environment to living organisms. From soil, plants adsorb and accumulate heavy metals. Through the food chain, heavy metals enter the animal kingdom including humans and cause health risks. Few physicochemical and phytoremediation approaches have been proved effective in removing heavy metals from contaminated soils. However, soil characteristics and recycling of soil constituents have made their practicability questionable. One pragmatic way to reduce the deleterious effect of heavy metals in soil is rhizoremediation, in which plant–microbe interaction is explored for remediation purposes. In this strategy, the plant growth-promoting rhizobacteria (PGPR) either accumulate or detoxify the heavy metals and thereby prevent the uptake and accumulation of heavy metals in plants. In addition, PGPRs act as biofertilizer that enhance the crop yields in different ecological niches. In this chapter, rhizoremediation strategy is described and portrayed as the pragmatic way for remediation of heavy metals in soil.

9.1   Introduction

Heavy metal, the poorly defined term, is the subset of 40 elements including transition metals, metalloids, lanthanides, and actinides (Appenroth 2010) that have specific density of more than 5 g/cm^3. They possess metallic characteristics such as ductility, conductivity, stability as cations, ligand specificity, etc. Though the term heavy metal is announced as a meaningless term by IUPAC, it is widely

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used in biology (Duffus 2002). Biologically, they are classified as essential and nonessential heavy metals. Some of the heavy metals like cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), and molybdenum (Mo) are essential elements whose role in metabolism is known in both prokaryotes and eukaryotes. In contrast, no nutritional function is known for heavy metals like silver (Ag), cadmium (Cd), arsenic (As), lead (Pb), mercury (Hg), and uranium (U) (Appenroth 2010). While in case of chromium (Cr), the role of Cr^{3+} ions in sugar and lipid metabolism is known, but the role of Cr^{6+} ions is unknown (Vincent 2000).

The discharges of heavy metals from natural and anthropogenic activities cause the accumulation of metals into the environment. Natural input includes withering and erosion of parent rocks that transfer large quantities of metals to water bodies and lands (Gadd 2010). As per the World Health Organization (WHO) report (1992), 15,000 metric tons (mt) of Cd is added to the oceans every year. A higher level of heavy metal accumulation is reported in the marine sedimentary rocks, marine phosphates, and phosphorites. Volcanic eruptions (Hong et al. 1996) and forest fires (Shcherbov et al. 2008) also contribute to natural inputs. In addition, the use of heavy metals by humans is known for years (Nriagu 1996). The uses include mining, smelting, fuel combustion, synthetic fertilizers, metal alloys, electroplating, and Ni–Cd batteries, as pigment in plastics and as stabilizer in PVC, in electronic goods, and in solar cells (Gadd 2010). Because of these activities, the contents of Pb, Hg, and Cd in the pedosphere (earth's outermost soil layer) are about 10, 6, and 5 times, respectively, higher than in the lithosphere (earth's crust and outermost mantle layer) (Han et al. 2002). Here, we summarize the toxicity of heavy metals, different approaches employed for soil remediation, and the distinctive properties of rhizoremediation approach, which have made this technology user-, eco-, and economic friendly.

9.2   Soil Pollution

Soil is the major reservoir for most of the metals and nonmetals including the nutrients and is the prime site of biogeochemical cycling of elements. Over the years, continuous cropping and other agricultural activities have resulted in depletion of nutrients in soil (Zhang et al. 2006), requiring application of plant nutrients from external sources. In this context, synthetic agrochemicals especially phosphate fertilizers are excessively applied, which have resulted in heavy metal pollution of soil (Mortvedt 1996; McGrath and Tunney 2010). Besides these, sewage sludge application and industrial activities also add heavy metals to agricultural soil (Kelly et al. 1999; Han et al. 2002). The major factors influencing metals speciation, adsorption, and distribution in soils include pH, soluble organic matter, hydrous metal oxide, clay content and type, organic and inorganic ligands, and competition from other metal ions (Dube et al. 2001).

Even though heavy metals in soil seem to be immobile, they interact with biotic components especially the plant roots. Thereafter, they are transported to all parts of the plants including the fruits, vegetables, and seeds. Consequently, heavy metals

enter the food chain of animals including humans (Fig. 9.1). It is reported that terrestrial foods account for 98% of the ingested toxic heavy metals, while 1% each of aquatic foods and drinking water (Van Assche 1998). The Joint Food and Agricultural Organization (FAO)/WHO Expert Committee on Food Additives (JECFA) determined the provisional tolerable weekly intake (PTWI) for the toxic heavy metals which is listed in Table 9.1. Among the toxic heavy metals, Hg and Cd have PTWI values less than 10 μg/kg body weight showing their high toxicity, while Cu and Zn being the essential metal ions have PTWI values of 3,500 and 7,000 μg/kg body weight, respectively. Various studies reported the presence of heavy metals higher than the PTWI values in vegetables, canned foods, and other eatables in all parts of the world. The technical report of Imperial College of London prepared in collaboration with Indian universities has revealed the heavy metal contamination of vegetables in Delhi, India (Marshall et al. 2003). According to the Indian Council of Medical Research (2003) report, nearly 50% of the tested mother's milk samples had Cd eight times more than stringent limits. Since heavy metals are not quickly eliminated from the human system, they bioaccumulate to
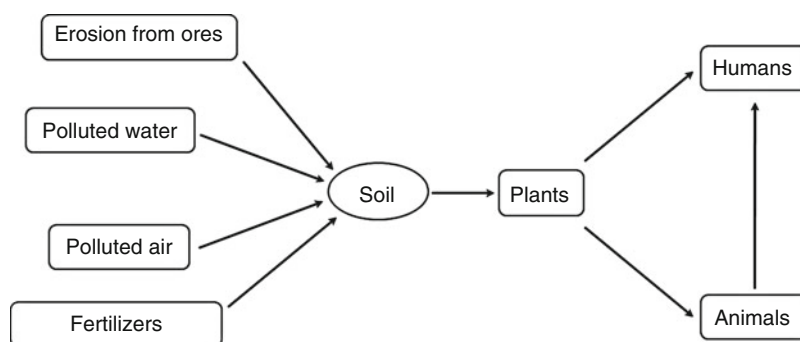
Fig. 9.1 Food chain exhibiting the transport of heavy metals

Table 9.1 Provisional tolerable weekly intake (PTWI) for heavy metals

Heavy metals	PTWI (μg/kg body weight)
Mercury	4
Cadmium	7
Arsenic	21
Chromium	23.3
Lead	25
Nickel	35
Silver	50
Copper	3,500
Zinc	7,000

Reports of Joint Food and Agricultural Organization (FAO)/WHO expert committee on food additives (JECFA)

toxic levels. The biological half-life of toxic heavy metals ranges between 20 and 30 years (Sugita 1978). The WHO (1987) estimated that the daily intake of 200 μg Cd for longer period can be connected with a 10% prevalence of adverse health effects suggesting threat to food security.

9.3 Heavy Metal Toxicity

Most of the heavy metals cause changes in both the environment and living organisms. Besides the nonessential heavy metals, even the essential heavy metals become toxic, when their level exceeds the physiological value. The prime reason for their toxicity is due to their ability to bind strongly to oxygen, nitrogen, and sulfur atoms because of free enthalpy of the metal–ligand product (Weast 1984). As a result, heavy metals inactivate the enzymes by binding to –SH group, leading to changes in metabolism (Fuhrer 1982). Many enzymes and proteins need essential divalent cations like Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}, which are displaced by the toxic divalent ions. For instance, in case of calmodulin, the protein that is important in cell signaling, the Ca^{2+} ions are displaced by Cd^{2+} ion, leading to loss of activity (Rivetta et al. 1997). The binding ability of heavy metals with nucleic acids allows them to act as mutagens, which lead to misreading of the genetic profile (Wong 1988). In addition, they cause lipid peroxidation and oxidative stress, leading to membrane damage (Howlett and Avery 1997).

Sources of heavy metals for plants are the soil, irrigation water, and air emissions. Plants take up heavy metals primarily from the soil and accumulate in the plant tissues (Fig. 9.2a). Following accumulation, heavy metals cause changes in the metabolic pathways like photosynthesis (Clijsters and Van Assche 1985; Somasundaram et al. 1994; Pandey and Tripathi 2011), protein and nitrogen metabolism (Hemalatha et al. 1997; Llorens et al. 2001; Manios et al. 2002; Priti et al. 2009), uptake of nutrients
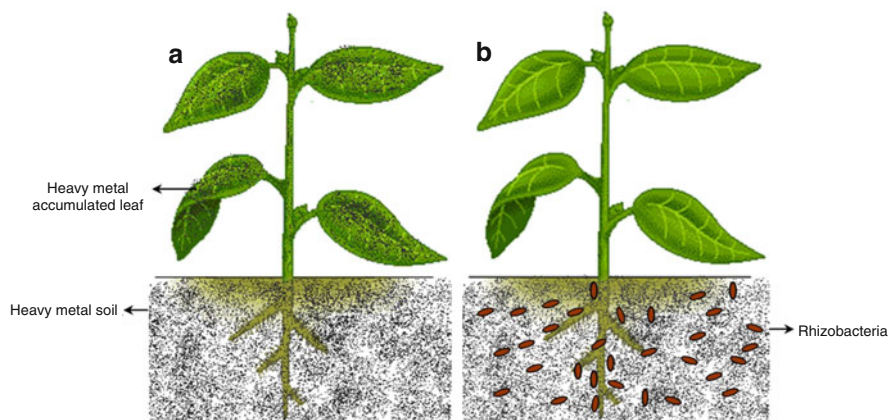
Fig. 9.2 Schematic representation of rhizoremediation

(Veselov et al. 2003), and sugar and water metabolism (Pandey and Tripathi 2011; Stobrawa and Lorenc-Plucińska 2007; Babula et al. 2008). Heavy metals also lead to hormonal imbalances and especially elevate ethylene synthesis (Arteca and Arteca 2007) and decrease cytokinin level due to oxidation (Hare et al. 1997). These effects lead to poor crop productivity. For humans, the heavy metals enter through polluted food, water, air, and occupational exposure. Heavy metals are carcinogens that alter the gene expression, leading to cell proliferation by the induction of proto-oncogenes or by interference with genes involved in cell growth (Beyersmann 2002). International Agency for Research on Cancer (IARC) grouped Cd, Cr, and As in group 1 (proven human carcinogens) and Ni, Co, Hg, and Pb in group 2B (possibly carcinogenic to humans). The teratogenic effects of heavy metals like Cd, Pb, Hg, and U are also reported (Emmanouil-Nikolussi 2007). The other health risks include renal tubular damage, bone demineralization, cardiac failure, nervous, respiratory disorders, and loss of fertility (Duruibe et al. 2007). Even low levels of Cd, Pb, and Hg exposure are reported to diminish intellectual capacity and brain development of children (Drum 2009).

9.4 Soil Remediation Approaches

Many different physical, chemical, and biological methods are proposed for the remediation of metal-contaminated soil.

9.4.1 Physicochemical Methods

The conventional *ex situ* methods like land filling, incineration, leaching, and chemical methods are adopted to remediate metal-contaminated soils, but they are not effective (Lambert et al. 2000). Other *in situ* approaches like vitrification and electrokinetics that involve the application of electrical voltages are efficient, but labor safety and cost factors are of major concern (Mulligan et al. 2001). All these techniques just transfer the contaminants from soils to some other material, which needs to be transported and recycled. Hence, the low efficiency, high cost, safety problems, recycling, transfer, and need to analyze the nature of the contaminants and type of soil are the major setbacks for these approaches. In addition, the ex situ modes and transfer of absorbed material in other techniques pose possibilities of spread of pollutants during transport. Furthermore, the nonbiological methods disrupt the soil characteristics and ecology that make the land unsuitable for agriculture and other purposes.

9.4.2 Bioremediation

9.4.2.1 Phytoremediation

Remediation of soil contaminated with hazardous substances utilizing the innate capabilities of plants is generally termed phytoremediation, an in situ eco-friendly and perpetual approach which does not require any specialized equipments. In addition, application of plants for abatement/rehabilitation of heavy metal-stressed soil is indeed a promising but emerging area of interest because it is an ecologically sound and environmentally safe method for restoration of degraded lands. In this context, about 0.2% angiosperms (Baker and Brooks 1989) are reported to tolerate and accumulate excessively high concentrations of metals and are often termed hyperaccumulators. Plants like *Alyssum* species, *Brassica juncea, Arabidopsis halleri, Noccaea* sp. (formerly *Thlaspi* sp.), *Viola calaminaria*, and *Astragalus racemosus* are hyperaccumulators. The molecular mechanism underlying hyperaccumulation is attributed to the involvement of metal-specific transporters, chelators such as phytochelatins (PC), metallothioneins (MT), and organic acids (OA) like citrate and antioxidants like glutathione (Kramer 2010). Phytoremediation as a technique broadly involves (1) phytoextraction: uptake and accumulation of metals in plants; (2) rhizofiltration: roots absorb, concentrate, or precipitate the metals; (3) phytostabilization: plant reduces the heavy metal mobility by precipitation; (4) phytodegradation: the pollutants are taken up by plants and degraded by the plant enzymes; and (5) phytovolatilization: uptake and release of metals into air as volatile compounds (Raskin and Ensley 2002). These characteristics are commonly associated with only hyperaccumulators, and hence, the normal plants are genetically engineered by introducing the genes involved in metal chelation, transport, and stress responses (Susan and D'Souza 2005). For example, engineering of human MT and mouse MT genes in different plants like *Arabidopsis*, tobacco, and rapeseed plants has shown enhanced Cd uptake and accumulation (Misra and Gedamu 1989). Thus, phytoremediation is a promising option, but longer time, climatic conditions, recycling of accumulated plants, and soil characteristics are some of the major constraints. In addition, the use of transgenic plants poses many unanswered ecological questions. Therefore, the better strategy that answers the problems with the above strategies is achieved by rhizoremediation that combines the advantages of plant–microbe symbiosis.

9.4.2.2 Rhizoremediation

Rhizosphere is defined as the soil zone of biological activity around the plant roots, which is the sink of nutrients. In this region, an intense interaction between plant roots and microbes takes place. Microbes inhabiting this area are generally termed rhizobacteria and regulate the biogeochemical cycles, degrade organic materials, and preserve the soil chemistry (Haferburg and Kothe 2007). The proven traits for effective root colonization include the synthesis of the O-antigen of lipopolysaccharide and cellulose, thiamine and biotin production, amino acid synthesis, an isoflavonoid inducible efflux pump, and a nine-polar flagellar arrangement (Lugtenberg et al. 2001). Using in vivo expression technology (IVET), about 20 genes were demonstrated to be induced in root-colonizing pseudomonads

(Silby and Levy 2004). Around the roots, they form microcolonies, often called biofilms which are covered by mucoid layer (Lugtenberg and Kamilova 2009).

One way to reduce the deleterious effects of heavy metals taken up from the environment by some plants involves the use of plant growth-promoting rhizobacteria (Khan et al. 2009) and mycorrhizae (Heggo and Angle 1990; Saraswat and Rai 2011), and this strategy is termed rhizoremediation. More precisely, the rhizoremediation is defined as the biological treatment of organic or inorganic contaminants in soils by bacterial or fungal activity in the rhizosphere (Kuiper et al. 2004). All through rhizoremediation, low- (e.g., phenolics, organic acid) and high-molecular-weight (e.g., proteins) exudates released from growing plants stimulate the viability and functionality of the plant growth-promoting rhizobacteria (PGPR), which consequently results in a more efficient transformation/degradation of environmental pollutants. The microbial activity also prevents the uptake and accumulation of heavy metals in different organs of plants (Fig. 9.2b). In general, the heavy metals are, however, toxic to the microbes, which in turn affect the fertility of soil. As a survival strategy, some microbes have evolved resistance/avoidance mechanisms that cause change in metal speciation (White et al. 1997). The strategies include biosorption of heavy metals by cell walls, polysaccharides, and pigments (Gadd 2009) and removal by the efflux pumps and by the synthesis of metal-binding peptides and proteins like MTs (Silver 1996). Thus, the microbial biomass acts as sink for the toxic heavy metals (Gadd 2010). In addition, some microbes detoxify the heavy metals like Cd, Hg, and Pb by enzymatic action (Aiking et al. 1985). Various studies reported the successful rhizoremediation of heavy metals in soil using heavy metal-resistant rhizobacteria and different plant species (Table 9.2). In heavy metal soil, the metal-resistant rhizobacteria also enhance the mycorrhizal and nodulation efficiency in plants (Vivas et al. 2006). Besides native bacteria, transgenic approaches both at the microbial and plant levels are reported for higher efficiency of rhizoremediation. For example, the expression of metal-binding peptide (EC20) in rhizobacteria *Pseudomonas putida* 06909 improved both cell growth and cadmium binding in the presence of the cadmium in sunflower seedlings (Wu et al. 2006). It was shown that the introduction of genetically modified microorganisms designed for rhizoremediation induces changes in native bacteria in the rhizosphere but not in the surrounding soil (Carcer et al. 2007).

9.5 Plant Growth-Promoting Activities of PGPR

Besides the heavy metal accumulation or detoxification, the plant-growth-promoting activities of rhizobacteria improve the efficiency of rhizoremediation. The PGPR promote the plant growth by hormonal regulation, enhanced mineral uptake, sequestering iron by siderophore production, antagonism by antibiotics production, and root growth stimulators production (Fig. 9.3) (Khan et al. 2009; Lugtenberg and Kamilova 2009; Martínez-Viveros et al. 2010; Ahemad and Khan 2011). Studies with metal-sensitive PGPR have also been found effective in preventing the accumulation

Table 9.2 Some examples of rhizoremediation of heavy metals using plant growth-promoting rhizobacteria

Rhizomicrobe	Microbial characteristics[a]	Heavy metal	Plant	Effect on plants[a]	Reference
Bacillus sp. PSB10	Chromate reducer; produced siderophore, IAA, HCN, ammonia, and solubilized insoluble P	Cr	*Cicer arietinum*	Increased growth, nodulation, chlorophyll, leghemoglobin, seed yield, and grain protein and decreased Cr accumulation	Wani and Khan (2010)
Serratia sp. SY5	Produced IAA and siderophore	Cd, Cu	*Zea mays*	Increased root biomass	Koo and Cho (2009)
Mesorhizobium sp. RC3	Chromate reducer; produced IAA and fixed N	Cr	*Cicer arietinum*	Increased nodulation, dry-matter content, seed yield, and grain protein; higher N content in roots and shoots; and decreased Cr accumulation	Wani et al. (2009)
Pseudomonas aeruginosa MKRh3	Cd^{2+} resistant; produced ACC deaminase, siderophores, IAA, and P	Cd	*Vigna mungo*	Enhanced plant growth with decreased Cd accumulation	Ganesan (2008)
P. aeruginosa KUCd1	Rifampicin-resistant mutant, Cd^{2+} tolerant, produced siderophore	Cd	Pumpkin, *B. juncea*	Enhanced growth with high chlorophyll and iron content and decreased Cd accumulation	Sinha and Mukherjee (2008)
Methylobacterium oryzae CBMB20 and *Burkholderia* sp. CBMB40	Methylotrophic, Ni^{2+}, and Cd^{2+} resistant	Ni, Cd	*L. esculentum*	Enhanced growth, decreased ethylene emission, and low Ni, Cd uptake	Madhaiyan et al. (2007)
Bradyrhizobium japonicum Cb1809	Produced nitrogenase and phytohormones	As	*Glycine max*	Enhanced dry weight and N content. Decreased As uptake	Reichman (2007)
Brevibacillus B-I	Zn^{2+}-resistant and produced IAA	Zn	*Trifolium repens*	Stimulated mycorrhization and nodulation. Increased biomass, P, and N uptake	Vivas et al. (2006)
P. fluorescens PRS9 Hg[r] and GRS9 Hg[r]	Hg^{2+}-resistant mutant; produced IAA, siderophore, and solubilized P	Hg	*Glycine max*	Enhanced plant growth	Gupta et al. (2005)
O. intermedium CrT-2, CrT-3, and CrT-4	Reduced Cr (VI) to Cr (III)	Cr	*Helianthus annuus*	Enhanced germination and plant growth. Increased auxin content and decreased Cr content	Faisal and Hasnain (2005)
Kluyvera ascorbata SUD165	Resistant to Ni^{2+}, Cd^{2+}, Zn^{2+}, CrO_4^{-}; displayed ACC deaminase activity	Ni, Pb, and Zn	*L. esculentum*, *B. juncea*, and *B. campestris*	High yield. Enhanced chlorophyll and protein content in leaves. Decreased Ni, Pb, and Zn uptake	Burd et al. (1998)

[a]Includes only reported parameters, but other characteristics might be absent or not tested

and toxicity of heavy metals in plants (Table 9.2). The different PGPR activities are discussed below in correlation with heavy metal toxicity in plants.

9.5.1   Heavy Metal Stress Tolerance

Primarily, heavy metal stress may cause hormonal imbalance in plants, leading to reduced root growth. Ethylene synthesis, for example, is increased upon treatment with Cd, Cu, and Zn. In the case of Cd and Cu, this increase is due to an upregulation of ACC synthase transcription and enhanced activity (Waldemar 2007). The microbial enzyme 1-aminocyclopropane carboxylic acid (ACC) deaminase catabolizes the immediate ethylene precursor ACC (Fig. 9.3), which is released in the root exudates. Thus, rhizobacteria acts as a sink for ACC by stimulating plants to exude more ACC and thereby reducing ethylene stress in plants (Penrose and Glick 2001). On treatment with Cd, the abscisic acid (ABA) hormone content rapidly increased in rice (*Oryza sativa*) seedlings (Hsu and Kao 2003). Secretion of cytokinin by the microbes decreases the ABA content and its effects (Cowan et al. 1999). In addition, PGPR also produce antioxidants like catalases and pyrroloquinoline quinine (PQQ) that helps in degrading the reactive oxygen species (ROS) which is synthesized during stress conditions (Fig. 9.3) (Yang et al. 2009).
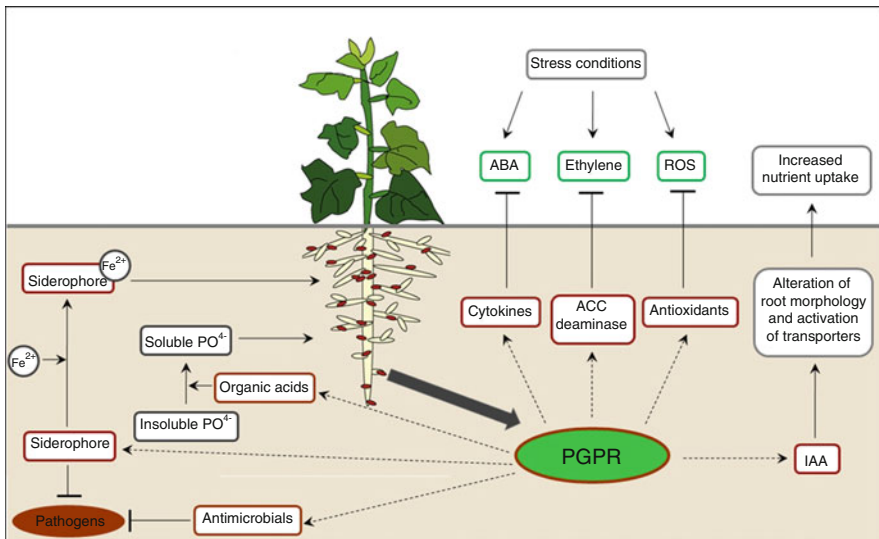
Fig. 9.3 Plant growth-promoting activities of rhizobacteria

9.5.2 Mineral Uptake

Various studies indicated the reduced uptake of minerals like iron, phosphate, nitrate, and other nutrients by plants grown in soils contaminated with heavy metals (Rubio et al. 1994; Huang et al. 2007). The deficiency of such elements in plants results in different types of symptoms on plants. For example, leaf chlorosis is one of the key morphological effects of heavy metals in plants due to iron starvation. Siderophores, the low-molecular iron-chelating substances produced by rhizobacteria, help in sequestration of iron by both microbes and plants (Neilands 1995). Siderophore-overproducing mutant of *Kluyvera ascorbata* SUD165, for example, enhanced the plant growth, chlorophyll contents in foliage, and protein content and decreased the heavy metal accumulation in tomato plants grown in metal-contaminated soil (Burd et al. 2000). Siderophore production is reported to be induced by heavy metals like Cd, Pb, Al, and Zn in *Pseudomonas* and *Rhizobium* spp. (Roy and Chakrabartty 2000; Sinha and Mukherjee 2008; Ganesan 2008), but the molecular mechanism underlying the synthesis of siderophore is not well explained.

Heavy metal ions also disrupt some of the important plant enzymes like nitrate and nitrite reductases, glutamine synthetase, glutamate synthase, and glutamate dehydrogenase (Llorens et al. 2001), leading to reduced uptake of ammonium and nitrate and low-protein content. This effect was circumvented by PGPR like Rhizobia and diazotrophs capable of producing nitrogenase. Due to these activities, legume–Rhizobia symbiosis has shown higher efficiency in rehabilitation of heavy metal-poisoned soils (Pajuelo et al. 2008; Wani et al. 2009). Similarly, phosphate-solubilizing microbes and arbuscular mycorrhizal fungi (AMF) enhance the P uptake in plants (Khan et al. 2007; Zaidi and Khan 2007; Zaidi et al. 2009; Lugtenberg and Kamilova 2009). Besides these, the production of hormones like indole acetic acid (IAA), cytokinin, and other metabolites (Fig. 9.3) promotes the root growth, modifies the root architecture, and induces the membrane transporters, which leads to the enhanced nutrient uptake by plants (Waldemar 2007).

9.6 Eco-economics

In spite of the billions of funding and development of newer technologies and programs aimed at restoring heavy metal-polluted soils, the severity of heavy metal problems is increasing alarmingly every year around the world. This is partly due to the lack of awareness but largely due to economic constraints mostly in developing countries. However, when applied, the comparative estimates and additional factors involved in remediation of metals, for example, cadmium per ton of soil (Glass 1999) employing various approaches, are presented in Table 9.3. The cost given in this table suggests that the rhizoremediation approach when employed properly with sound understanding is inexpensive. The estimates shown here include only the remediation cost, while the other expenses like cost of transport, recycling, and monitoring may further increase the overall cost of remediation. Further, since

Table 9.3 Estimates of cost for cadmium remediation per ton of the soil by different approaches

Remediation approaches	Estimates (US $/ton)	Additional factors and expenses
Land filling	100–500	Transport/excavation
Vitrification	75–425	Long-term monitoring
Chemical treatment	100–500	Recycling
Electrokinetics	20–200	Monitoring
Phytoremediation	5–40	Recycling and monitoring
Rhizoremediation	5–20	Monitoring

Adapted from Glass (1999)

rhizoremediation approach involves the use of cheap renewable resources like PGPR having multiple properties, this technology could be more profitable than other remedial technology. The biocontrol activities like antagonism and competition for nutrients and niches (CNN) (Lugtenberg and Kamilova 2009) add further strength to the economic friendliness of rhizoremediation approach by cutting off the costs for pesticides and thereby circumventing phytopathogens naturally. Thus, rhizoremediation approach is made environmentally as well as economically more pragmatic.

Conclusion

Rhizoremediation approach is aesthetically pleasing and low cost, uses solar energy, requires minimal maintenance, presents no need for further recycling, and preserves the soil fertility and ecology. As a result, this strategy is gaining wider acceptance. Besides remediation and earning, it ensures the food security for humans and prevents them from a lot of ailments. However, large-scale field trials and its assessment are required to guarantee the practicability of rhizoremediation. However, how this technology could be useful in the rehabilitation of metal contaminated but nonagricultural soils with poor nutrients or nutrient deficient soils is indeed a challenge before scientists. Considering different facets of remediation methods, it is evident that all these methods in general provide only a temporary solution for the abatement of polluted lands and not complete destruction of metals from the contaminated sites. Hence, the practice of organic farming along with the remedial technology should be promoted in order to prevent metal pollution in agricultural soil.

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Role of Plant-Growth-Promoting Rhizobacteria in the Management of Cadmium-Contaminated Soil

10

Ashok Kumar

Abstract

During the last decades, heavy metals have become a common contaminant worldwide. Root-colonizing bacteria that exert beneficial effects on plant development directly or indirectly, often called as plant-growth-promoting rhizobacteria (PGPR), play an important role in the remediation of heavy-metal-contaminated soils. The prospect of manipulating rhizosphere microbial populations by inoculating beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable under the field trials. In addition to their role in metal decontamination/removal, PGPR have also been found to facilitate plant growth in conventional soils by various mechanisms. These mechanisms include the suppression of phytopathogens by producing siderophores, synthesizing antifungal antibiotics, secreting fungal cell-wall-lysing enzymes, or hydrogen cyanide in addition to the release of growth-promoting hormones, solubilization of insoluble phosphate, and providing other essential nutrients to plants. Here in this chapter, the role of PGPR in metal especially cadmium decontamination is highlighted.

10.1 Introduction

Heavy metals are continuously added to soils through various agricultural and industrial activities. Such activities include the use of agrochemicals and the long-term application of sewage sludge, waste disposal, waste incineration, and vehicle exhausts. These activities lead to accumulation of obnoxious elements in

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agricultural soils and pose a threat to food safety and potential health risks (Jing et al. 2007). In addition, heavy metals have received increasing attention in recent years due to adverse impact on microbial compositions and their associated activities, soil fertility, plant development, and directly or indirectly human health (Khan et al. 2009). Among heavy metals, cadmium is highly mobile in soils and is the most toxic metal. When taken up by plants, cadmium inhibits the growth of plant organs like root and shoot, affects nutrient uptake and homeostasis, and accumulates in important crops which could later on be consumed by animals and humans (Sanita di Toppi and Gabrielli 1999). Cadmium contamination also negatively affects the diversity and the activity of agronomically important soil microbial communities (Roberts 2003; Lenntech 2009; Shukla et al. 2010). The highest amount of cadmium enters into the soil through application of sewage sludge and wastes disposal. In addition, cadmium also enters into soil from various industrial activities such as dye making, rubber making, production of fertilizer from phosphate rock, automobile fuel, and metal-melting industry. The heavy metals, however, cannot generally be biologically degraded to more or less toxic products and persist in the environment. The threat of heavy metal pollution has, therefore, led to an increased interest in developing systems that could remove or neutralize the toxic effects of metals found in soils, sediments, and wastewaters. In this context, some microorganisms, like *Bacillus subtilis*, *Citrobacter* spp., and *Pseudomonas* spp., and plants, like *Trifolium repens*, *Brassica napus*, *Salix viminalis* (Willow), *Thlaspi caerulescens*, and *Populus canadensis*, have been found effective in reducing/remediating the toxicity of metals including cadmium (Sell et al. 2005; Sheng and Xia 2006; Frerot et al. 2006; Ganesan 2008; Sheng-wang et al. 2008; Chunxiao et al. 2009).

Green plants proposed for in situ soil phytoremediation (Brooks 1998; Salt et al. 1995) have now become an attractive topic of research and development for environmentalists. Plant-assisted bioremediation, or phytoremediation, is commonly defined as the use of green or higher terrestrial plants for treating chemically or radioactively polluted soils. Even though some workers have quantified and compared the role of soil microbial communities in phytoremediation of polycyclic aromatic hydrocarbons (PAHs) (Walter 2009; Johnson et al. 2005), the use of phytoextraction strategy in removing heavy metals from contaminated environment has become increasingly attractive despite the fact that heavy metals can be toxic to even metal-accumulating and metal-tolerant plant. Another possibility to lessen the deleterious effects of heavy metals onto plants could be the use of PGPR (Belimov et al. 2005; Khan et al. 2009) which are defined by three intrinsic characteristics: (1) they must be able to colonize the root, (2) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities, and (3) they must promote plant growth (Espinosa-Urgel 2004; Gamalero et al. 2004; Zahir et al. 2004; Ashrafuzzaman et al. 2009; Mishra et al. 2010; Martínez-Viveros et al. 2010; Ashrafi and Seiedi 2011).

10.2   Cadmium Features

The pollution of the environment with toxic heavy metals including cadmium is spreading throughout the world along with industrial progress (FWPCA 1968). Cadmium (Cd; at. no. = 48; at. wt. = 112.4) is a soft, silvery bluish-gray metal that is malleable and ductile transition metal, similar to zinc. It is soluble in acids but not in alkalis. About three fourths of cadmium is used in Ni–Cd batteries, most of the remaining one fourth is used mainly for pigments, coatings, and plating, and as stabilizers for plastics. Cadmium has been used particularly to electroplate steel where a film of cadmium only 0.05 mm thick will provide complete protection against the sea. Cadmium has the ability to absorb neutrons, so it is used as a barrier to control nuclear fission (Lenntech 2009). In nature, essentially all cadmium exists as seven stable isotopes and one radioactive isotope. The seven stable isotopes and their approximate abundances are cadmium-106 (1.3%), cadmium-108 (0.9%), cadmium-110 (12%), cadmium-111 (13%), cadmium-112 (24%), cadmium-114 (29%), and cadmium-116 (7.5%). The primary radioactive isotope, cadmium-113, comprises about 12% of natural cadmium and has an extremely long half-life. Nine major radioactive isotopes of cadmium exist, of which only three—cadmium-109, cadmium-113, and cadmium-113 m (metastable)—have half-lives long enough to warrant potential concern. The half-lives of the other six are less than 45 days. Cadmium-109 decays by electron capture with a half-life of 1.3 years, so any that was produced more than 20 years ago has long since decayed away. The other two cadmium isotopes decay by emitting a beta particle. The very low specific activity of cadmium-113 limits its radioactive hazards. Cadmium-113 m decays by emitting a beta particle with no gamma radiation (ANL USD 2005).

10.3   Source of Cadmium

10.3.1 Soil

Cadmium in soils is derived from both natural and anthropogenic sources. Natural sources include underlying bedrock or transported parent material such as glacial till and alluvium. Cadmium can be transported over great distances when it is absorbed by sludge. The cadmium-rich sludge can pollute surface waters as well as soils. Cadmium strongly adsorbs onto organic matter in soils. The cadmium in soils occur at very low levels, but its concentration in soil increases following application of cadmium-containing materials like fertilizers, phosphogypsum, certain zinc additives, biosolids (sewage sludge), manures, and other wastes. Cadmium is much less mobile in soils than in air and water. The major factors governing cadmium speciation, adsorption, and distribution in soils are pH, soluble organic matter content, hydrous metal oxide content, clay content and type, presence of organic and inorganic ligands, and competition from other metal ions (OECD 1994). Atmospheric cadmium emissions' deposition onto soils has generally decreased significantly over that same time period (Cook and Morrow 1995;

Table 10.1 Cadmium levels in ores and rocks (mg/kg)

Igneous, metamorphic, and sedimentary rocks	Zinc ores	Ores for iron and steel	Cement material	Fossil fuels	Phosphate fertilizers	Copper and lead ores
0.02–0.25	200–14000	0.1–5.0	2.0	0.5–1.5	10–200	~500

Adapted from http://www.cpcb.nic.in/oldwebsite/News%20Letters/Latest/cadmium/ch8-CAD-MIUM.htm

Mukunoki and Fujimoto 1996). Once accumulated within soil system, cadmium remains there for longer time periods which may take about 100–1,000 years for leaching of cadmium from the soil to half (CPCB 2007). The concentrations of cadmium found in different rocks and ores are presented in Table 10.1.

Cadmium in nonagricultural soils generally does not affect human health because it may not enter the human food chain, but its indirect transfer from nonagricultural soil to agricultural soils via airborne or water transport may affect the food chain and consequently the human health. Cadmium in agricultural soils is relatively immobile but become mobile under acidic conditions. The availability of cadmium in soil is controlled largely by the pH, and its mobility increases with decreasing pH of the soil (CPCB 2007). The average value of cadmium in the earth's crust ranged between 0.1 and 0.5 mg/kg (ppm), but this may vary greatly depending on a large number of factors. For example, igneous and metamorphic rocks may have 0.02–0.2 mg/kg, whereas in sedimentary rocks it could be 0.1–25 mg/kg. Naturally, sulfide and oxide ores of zinc, lead, and copper contain even higher levels such as 200–14,000 mg/kg for zinc ores and about 500 mg/kg for typical lead and copper ores. The raw materials used in iron and steel production contain approximately 0.1–5.0 mg/kg, while those for cement production contain about 2 mg/kg. Fossil fuels contain 0.5–1.5 mg Cd/kg, but phosphate fertilizers contain from 10 to 200 mg Cd/kg (Jensen and Bro-Rasmussen 1992; OECD 1994; Cook and Morrow 1995; Mukunoki and Fujimoto 1996).

10.3.2 Compost and Vegetables

Cadmium in municipal solid waste (MSW) composts results from variety of sources like batteries, consumer electronics, ceramics, house dusts and paint chips, light bulbs, lead foils, used motor oils, plastics, and some inks and glasses. Composts made from these solids waste will inevitably contain cadmium. It is reported that MSW composts contain more cadmium than found in average soils (Lenntech 2009; Woodbury 2005; CPCB 2007). On the other hand, cadmium is taken up from soil by the plant roots. The plants grown in soils that are very sandy, acidic, and/or low in organic matter (OM) content absorb cadmium more easily and rapidly. Cadmium in soil attaches to clay particles and OM, sandy soils with low clay content, and OM induces higher uptake of cadmium. The availability of cadmium to plants however decreases as the soil pH increases. Likewise, higher concentrations of chloride in soil mobilize cadmium and increase uptake by plants.

After they enter plant systems, cadmium may be concentrated in various organs such as leaves, roots and tubers, seeds or grain, and fleshy fruits. Leafy vegetables such as lettuce, spinach, potatoes, and grain foods are reported to accumulate higher concentration of cadmium. And hence, vegetables like garlic (*Allium sativum*), carrot (*Daucus carota*), beetroot (*Beta vulgaris* var. *altissima*), spinach (*Basella alba*), silver beet (*B. vulgaris* var. *cicla*), pea (*Pisum sativum*), lettuce (*Lactuca virosa*), and cabbage (*Brassica oleracea*) are grouped under high risk of cadmium. In contrast, other vegetables like capsicum, tomato, cauliflower, mushroom, and alfalfa are placed in medium-risk level, while pumpkin, green bean, and cucumber come under low risk. The risk of cadmium to potato of various varieties comes under all the three categories viz. high, medium, and low risk (Lenntech 2009; CPCB 2007). The presence of cadmium in cow milk may possibly be due to the fodder containing higher level of cadmium, given to cows.

10.4   Cadmium Poisoning

Cadmium is reported to have no constructive role in the human body but is extremely toxic even at low concentrations when it accumulates inside organisms and/or agroecosystems. Buildup of cadmium levels in the water, air, and soil has been on the rise particularly in the industrial areas. Serious toxicity problems have resulted from long-term exposure to cadmium-plating baths. Cadmium causes renal tubular dysfunction after long-term exposure (Friberg et al. 1986). Environmental exposure to cadmium has been a serious problem for people of Japan where many people after consuming rice (*Oryza sativa*), grown in cadmium-contaminated irrigation water, suffered from a disease called itai-itai disease. Foods obtained from plant materials grown in cadmium contaminated soils, tobacco (*Nicotiana tabacum*), wastes discharged from industries, etc. are also a significant source of cadmium exposure. Workers associated with various industrial operations like smelting and refining of metals, soldering or welding, batteries, coatings, or plastics are also at cadmium toxicity risk (Elinder 1985; WHO 1992; Watanabe et al. 1993, 1994; OECD 1994; Lenntech 2009).

10.4.1   Clinical Effects of Cadmium

Acute exposure to cadmium fumes may cause flu-like symptoms, including chills, fever, and muscle ache, which is sometimes referred to as the cadmium blues. More severe exposures can cause tracheobronchitis, pneumonitis, and pulmonary edema. Symptoms of inflammation may start hours after the exposure and include cough, dryness, and irritation of the nose and throat, headache, dizziness, weakness, fever, chills, and chest pain (ANL USD 2005). Inhaling cadmium-containing dust may cause respiratory and kidney problems which can be fatal (often from renal failure). Cadmium, classified as a probable human carcinogen under the EPA 1996 cancer guidelines, may also cause immediate poisoning and damage to the liver and the kidneys.

Compounds containing cadmium are also carcinogenic. The bones become soft, e.g., osteomalacia, lose bone mineral density (osteoporosis), and become weaker. This causes the pain in the joints and the back and also increases the risk of fractures (Blainey et al. 1980). The proximal renal tubular dysfunction creates low phosphate levels in the blood (hypophosphatemia), causing muscle weakness and sometimes coma. The dysfunction also causes gout, a form of arthritis due to the accumulation of uric acid crystals in the joints because of high acidity of the blood (hyperuricemia), and suppression of erythropoietin blood (Horiguchi et al. 2000). Another side effect is increased levels of chloride in the blood (hyperchloremia). The kidneys can also shrink up to 30%. However, experimental studies suggest that cadmium could also interfere with the nervous system (Lafuente et al. 2003; Pohl et al. 2003). Chronic exposure may result in emphysema and chronic bronchitis. Repeated low exposures may also cause permanent kidney damage, leading to kidney stones and other health problems. In its narrative for the cancer weight of evidence, the US Environmental Protection Agency (EPA) states that occupational studies of cadmium smelter workers developing lung cancer provide limited evidence for the carcinogenicity of cadmium in humans following inhalation exposure and that there is sufficient evidence of carcinogenicity in rats and mice by inhalation and intramuscular and subcutaneous injection.

10.4.2 Cadmium Toxicity to Plants

Heavy metals including cadmium can induce essential nutrient deficiency and even decrease concentrations of several macronutrients in plants (Siedleska 1995). The crops grown in metal-contaminated soils often suffer from metal toxicity leading to losses in yields. Indeed, the toxic effects of cadmium on plant physiology are well documented (Vassilev et al. 2004). In fact, Cd has a low redox potential, and therefore it cannot participate in biological redox reactions, but there exists some evidence that it could perform oxidative-related disturbances, including lipid peroxidation (Sandalio et al. 2001). The negative impact of Cd on cell redox status is known and explained by the high affinity of cadmium ions to SH groups of proteins, which may affect their functional properties (Vangronsveld and Clijsters 1994). When plant cells are not able to maintain low free Cd ions in the cytosol through efficient detoxifying mechanisms, this may lead to depletion of the cell defense network and as a consequence to oxidative damages to important molecules, including lipids. Generally, many factors at different structural–functional levels may disturb the photosynthetic process in plants, grown in the presence of cadmium. However, the negative effect was probably due to the lower photosynthetic pigment content, as both stomatal conductance and transpiration rate were not significantly affected by cadmium (Vassilev et al. 2004).

10.5   Bioremediation and PGPR

The remediation of soils contaminated with heavy metals can be performed using chemical, physical, and biological techniques. Chemical and physical methods have the advantage of a short remediation time, but are expensive and cause secondary pollutants (Kumino et al. 2001). Among bioremediation, phytoremediation, the process of utilizing plants to absorb, accumulate, and detoxify heavy metals in soil, is considered an alternative strategy for the remediation of soils contaminated with heavy metals (Gerhardt et al. 2009; Ma et al. 2009a). This method is ecologically sound, safe, and cost effective, but its remediation efficiency is mostly affected by limiting factors, such as meteorological factors and the toxicity of pollutants (Gerhardt et al. 2009; Kumino et al. 2001). Plants used for extraction of metals from contaminated soil must be tolerant to heavy metals, adapted to the local soil and climate characteristics, and able to take up a large amount of metals (Keller et al. 2003). Generally, two groups of plant species are considered for metal phytoextraction: (1) hyperaccumulating species, able to accumulate and tolerate extraordinary metal levels, and (2) high-biomass-producing species, such as maize, tobacco, and sunflower, compensating moderate metal accumulation by high biomass yield (Mench et al. 1989; Kumar et al. 1995; Herzig et al. 2003; Vassilev et al. 2004).

The metal and organic pollutants can be removed by the microbial flora. *Bacillus* sp. was very much efficient to remove the Au, Cd, Cr, Fe, Mn, Ni, Pb, U, and Zn. It was recorded that *Bacillus* sp. can efficiently remove the metal pollutants from the waste or industrial effluents (Brierley and Brierley 1993; Philip et al. 2000; Gunasekaran et al. 2003). *Pseudomonas* sp. was also reported to Cu, Cr, Cd, Pb, Ni, U, and Zn (Kapley et al. 1999; Sar and D'Souza 2001; Cybulski et al. 2003; Tarangini 2009).

Selected microbes can degrade most environmental pollutants (Shukla et al. 2010). The process of pollutant degradation by microbes, however, ceases when the microbes are starved of foods. In order to ascertain that such microbes can have access to the best food source available in soil, namely, root exudates, workers have described an enrichment method for the isolation of microbes (Kuiper et al. 2001) which combine the properties of (1) degradation of a selected pollutant and (2) excellent root colonization. They have termed this process rhizoremediation instead of phytoremediation to emphasize the roles of the root exudates and the rhizosphere competent microbes. Plant root exudates such as sugars, alcohols, and organic acids act as carbohydrate sources for the soil microflora and enhance microbial growth and activity. Some of these compounds may also act as chemotactic signals for microbes. The plant roots also loosen the soil and transport water to the rhizosphere, thus additionally enhancing microbial activity (Kudjo 2007; Shukla et al. 2010).

However, there are reports that suggest that PGPR (e.g., *Pseudomonas putida* KT2440) among microbes could help to reduce the toxicity of heavy metals if they are applied as inoculant (Lázaro et al. 2000; Wani et al. 2007). Thus, well-equipped PGPR settle on the root together with the indigenous population and consequently enhance the bioremediation process. The rhizoremediation of

heavy-metal-contaminated soils has become important because polluted soils cover huge areas that are rendered unsuitable for agricultural production. To overcome metal stress, microorganisms have evolved variety of mechanisms like (1) pumping of metal ions exterior to the cell, (2) accumulation and sequestration of the metal ions inside the cell, (3) transformation of toxic metal to less toxic forms (Wani et al. 2008), and (4) adsorption/desorption of metals.

To promote the uptake efficiency of heavy metals by plants, many investigations have focused on the close relationship between plants and plant-growth-promoting rhizobacteria (PGPR). Some rhizobacteria can reduce the toxicity of heavy metals, resulting in the stimulation of plant growth (Black et al. 1993; Burd et al. 2000; De-Souza et al. 1999). They can excrete organic acids to enhance the bioavailability of heavy metals (Abou-Shanab et al. 2003). Several established studies indicated that PGPR can promote the growth of plants under the toxicity of Ni, Pb, or Zn (Burd et al. 1998, 2000; Grichko et al. 2000). In addition, PGPR have been reported as phytoextraction assistants: *Pseudomonas* sp. (Farwell et al. 2007; Sheng et al. 2008; Braud et al. 2009; Ma et al. 2009b), *Bacillus* sp. (Ma et al. 2009b; Sheng and Xia 2006), *Mesorhizobium* sp. (Ike et al. 2007), *Microbacterium* sp. (Abou-Shanab et al. 2006; Sheng et al. 2008), *Rhizobium* sp. (Abou-Shanab et al. 2006; Rai et al. 2004), *Variovorax* sp. (Belimov et al. 2005), *Rhodococcus* sp. (Belimov et al. 2005), *Psychrobacter* sp. (Ma et al. 2009a, b), *Flabobacterium* sp. (Belimov et al. 2005), *Sinorhizobium* sp. (Di Gregorio et al. 2006), and *Achromobacter* sp. (Ma et al. 2009a).

Recently, microbe-assisted phytoremediation has appeared as a more successful approach for the remediation of soils contaminated with heavy metals. Therefore, the exploration of new microbial resources, including PGPR, is still necessary for the development of in situ remediation strategies under multifarious conditions. Moreover, a better understanding of the interaction between PGPR and their host plants is important for enhancing the efficiency of microbe-assisted phytoremediation.

10.6 Importance of Plant-Growth-Promoting Rhizobacteria in Plant Growth

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria, like PGPR (Kloepper and Schroth 1978), to increase plant growth has shown considerable promise in laboratory and greenhouse studies. The response of such PGPR under field environment has, however, been variable (Bowen and Rovira 1999). The environmental benefits of using PGPR as microbial inoculants have been the reduction in the use of agricultural chemicals and its eco-friendly nature. Free-living as well as symbiotic PGPR can improve plant nutrition and growth, plant competitiveness, and responses to external stress factors (Egamberdiyeva and Hoflich 2004; Mantelin and Touraine 2004). Mishra et al. (2010) reported that the PGPR were efficient for the seed germination and plant growth of *Cicer arietinum* under salinity and can be used as biofertilizer.

PGPR can promote the growth of plants using direct and indirect mechanisms. Direct mechanisms include lowering the production levels of ethylene through synthesis of 1-aminocylopropane-1-carboxylate (ACC) deaminase in plants (Reed and Glick 2005; Safronova et al. 2006; Saleem et al. 2007); providing bioavailable phosphorus for plant uptake and atmospheric nitrogen fixation (Kloepper et al. 1980; Patten and Glick 1996) for plant use; sequestering trace elements like iron using siderophores (Glick 1995), e.g., *Kluyvera ascorbata* SUD 165 that has the ability to synthesize the enzyme ACC deaminase protected *Brassica juncea* and *Brassica campestris* against Ni, Pb, and Zn toxicity (Burd et al. 1998; Borgmann 2000) by reducing the stress caused by high ethylene level; and production of plant hormones like gibberellins, IAA, cytokinins, and auxins (Glick et al. 1999). Root elongation of *Brassica napus* has also been shown to be stimulated by IAA synthesized by PGPR (Sheng and Xia 2006) as well as nonidentified rhizobacteria on the *B. juncea* roots (Belimov et al. 2005).

PGPR that indirectly enhance plant growth by suppressing phytopathogens do so by a variety of mechanisms. These include the ability to (1) produce siderophores that chelate iron, making it unavailable to pathogens; (2) synthesize antifungal metabolites such as antibiotics, fungal-cell-wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; (3) successfully compete with pathogens for nutrients or specific niches on the root; and (4) induce systemic resistance (Bloemberg and Lugtenberg 2001; Glick 1995; Persello-Cartieaux et al. 2003; Martínez-Viveros et al. 2010) (Table 10.2).

10.7   Problems and Perspective of PGPR in Commercialization

Prior to registration and commercialization of PGPR products, numerous problems, like how quality, stability, and efficacy of the PGPR product can be preserved, requires attention. In addition, formulation development must consider factors such as shelf life, compatibility with current application practices, cost, and a proper delivery system. Health and safety are the other concerns that require special attention because these are the living organisms and hence should not be toxic, allergic, and pathogenic, persistence in the environment, and potential for horizontal gene transfer. The success of these products will however depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms (Bowen and Rovira 1999). Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculant formulation and delivery (McSpadden and Fravel 2002). Genetic improvement of PGPR strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion (Glick 1995; Bloemberg and Lugtenberg 2001; Lubeck et al. 2000). Genetic manipulation of host crops for root-associated traits to enhance establishment and proliferation of beneficial microorganisms (Smith and Goodman 1999; Mansouri et al. 2002) is being pursued. However, regulatory issues and public acceptance of genetically engineered organisms may delay their commercialization. The use of multistrain inocula of

Table 10.2 Effect of PGPR on different plants grown in cadmium-contaminated soil

PGPR	Plant	Effect(s)	References
Pseudomonas tolaasi ACC23, *P. fluorescens ACC9*	*Brassica napus*	The Cd content per plant did not increase significantly, but roots and shoots were increased by 83% and 94%, respectively	Dell'Amico et al. (2008)
Pseudomonas aeruginosa	Black gram	Decreased the accumulation of Cd in plants, showed extensive rooting, and enhanced plant growth	Ganesan (2008)
Pseudomonas sp. RJ10, *Bacillus* sp. RJ16	Rape (*Brassica napus*)	Increased uptake of Cd by plant and significantly enhanced shoot and root dry weight; increased shoot and root Cd content from 11% to 136% and 20% to 27% compared to control; increased shoot and root dry weight significantly	Sheng and Xia (2006)
Pseudomonas aeruginosa	Mustard and pumpkin	Improved growth and rooting; Cd uptake was reduced by 9% in pumpkin roots and 47% in shoots; Cd uptake was reduced by 52% and 37% in roots and shoots, respectively, of mustard	Sinha and Mukherjee (2008)
V. paradoxus 2C-1, 2P-1, 2P-4, *Flavobacterium* sp. 5P-4, *Rhodococcus* sp. 4N-4	Indian mustard (*Brassica juncea*)	Increased the length of roots significantly	Belimov et al. (2005)
Burkholderia cepacia	*Sedum alfredii*	Increased plant growth with Zn treatment up to 110%; increased Cd and Zn uptakes up to 243% and 96.3%, respectively	Li et al. (2007)
Burkholderia sp. J62	Maize and tomato	Increased the biomass of maize and tomato plants significantly; the increased Cd contents in tissues varied from 5% to 191%	Jiang et al. (2008)
Azospirillum lipoferum 137, *Arthrobacter mysorens* 7, *Agrobacterium radiobacter* 10	Barley cultivar Tselinnyi-5	Increased growth of plants and uptake of nutrients; prevented the accumulation of Cd	Belimov et al. (2004)
Bradyrhizobium sp., *Pseudomonas* sp., *Ochrobacterium cytisi*	*Lupinus luteus*	Decreased Cd accumulation. However, plant biomass was increased	Dary et al. (2010)

PGPR with known functions is of interest as these formulations may increase consistency in the field (Jetiyanon and Kloepper 2002; Siddiqui and Shaukat 2002). PGPR thus offer an environmentally sustainable approach to maintain soil fertility and concomitantly to increase crop production in various agroecosystems.

Conclusion

The increase of heavy metal pollution in the environment has led many researchers to focus on developing fast, economical, and more efficient remediation technologies. Indeed, rhizoremediation has been suggested as an

environmentally friendly technique. Extensive research in the areas of colonization capability, the role of rhizobacteria and plant roots in the uptake of metals, and their mode of metal translocation, is however required to further understand the mechanisms of PGPR which could provide protection to crops against metal toxicity and is likely to help to achieve the stabilization, revegetation, and remediation of metal-polluted soils.

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Site-Specific Optimization of Arbuscular Mycorrhizal Fungi Mediated Phytoremediation

11

Tünde Takács

Abstract

Anthropogenic contamination of soils with toxic metals has become a global environmental problem. Managed mycorrhization promotes phytoremediation and reuse of damaged fields. Site-specific optimization can be defined as selection of a tolerant fungal strain that is compatible to plants, remediation sites and the bioremediation method to be adapted. The high inter- and intraspecific functional diversity and non-specific association of arbuscular mycorrhizal (AM) fungi provide biological materials to develop fungi host combinations for different soils and contaminants. Both ecological and human health aspects should, however, be considered while planning and designing the phytotechnologies for restoration of metal contaminated sites. Soil characteristics, metal concentration, composition of the indigenous AM fungi and plant community are some of the important factors in developing site-specific remediation technology. The research carried out during the last few years on the role of AM fungi in facilitating phytoremediation of heavy metal contaminated soils under field environment is highlighted.

11.1 Soil Pollution and Its Treatment: A General Perspective

During the last decades, industrialized human activities have resulted in the significant pollution of environmental elements. Heavy metals (HMs) are common and perhaps more dangerous pollutants because they cannot be destructed biologically and, therefore, persist in the environment. Urban dust, soil, plant and animal tissue can accumulate them without visible signs, and uptake of HM through the

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food chain causes problems to human health. With regard to the variable nature of pollutant and contaminated environments, different factors should be considered while selecting the proper remediation technology. Such factors include (1) the remediation spot, (2) the quality and the quantity of the pollutant, (3) the depth of the pollution sites and (4) the characteristics of the soil. In this context, conventional physical and chemical remediation methods attempted have been found costly with damaging side effects and, therefore, are not preferred. Bioremediation on the other hand is the use of organisms for the treatment of damaged environment including soil polluted with contaminants. Among bioremediation, phytoremediation is a quickly flaring, environment friendly technology that supports sustainable development. Observing the vegetation in HM-polluted soils, phytoremediation, a method based on metal uptake of plants, was suggested in 1982. Botanical remediation is the mopping of polluted soil, sediment, water and sewage with natural or genetically modified, native or agricultural, terrestrial or water plants which have special metal uptake properties (Chaney et al. 1997; EPA 2001).

Plants used in phytoremedial soil cleaning can accumulate and sequester HMs in different tissues of root, stem or leaf. Compared to the conventional chemical methods, phytoextraction is an environmental-friendly and energy-saving procedure that employs renewable resources. Implementation and maintenance of this technology are cheap and aesthetic, and this method can be used both in situ and ex situ for a wide range of pollutants (Khan et al. 2009). The method can also be applied for moderately polluted soils where the aim is not to remove all the contamination but to reduce its concentration below threshold level (Cunningham and Ow 1996; McGrath et al. 2002). For larger polluted areas, phytoremediation is the only economically viable option. However, there are some disadvantages concerning phytoremediation. Firstly, it is a time-consuming, long-term process and can be applied only in the root activity zone. Metal uptake by plants differs among plant species and varies with metal species and climatic conditions. Since exogenous plant species can alter or destruct biodiversity, hence indigenous species should be preferred in phytoremediation technology (Turnau and Haselwandter 2002) which includes phytoextraction, phytodegradation, hydraulic control (phytohydraulics), phytostabilization, phytovolatilization, rhizodegradation and rhizofiltration (EPA 2001). While developing phytotechnology, the first aim should be to identify and select fast growing plants with massive biomass producing ability and having robust root system. Secondly, the growth of plants should be increased following fertilizer application or by applying mobilizing amendments. The phytoremediation process can be optimized with timely sowing of seeds, proper irrigation and by applying microbial inoculants. Soil microbial community is one of the key components of soil functionality and resilience as an important indicator of terrestrial ecosystem state (Szili-Kovács et al. 2007). A new and current development in bioremediation research is the application of mycorrhiza, the mutual fungus–plant symbiosis to enhance the removal or immobilization of the pollutants (Leyval et al. 2002; Hildebrandt et al. 2007; Göhre and Paszkowski 2006; Vosatka et al. 2006; Compant et al. 2010; Saraswat and Rai 2011). This chapter focuses on the challenges

as to how arbuscular mycorrhizal fungi (AMF) could be used to improve site-specific phytoremediation.

11.2 AM Fungi as Potential Tool for Phytoremediation

Mycorrhizae generally occur in most of the terrestrial ecosystems but can be absent only in the habitat with extreme soil conditions or disturbed, eroded and fumigated soils (Brundrett 2002; Trappe 1987). The most ancient and widespread mycorrhiza is the arbuscular one, which belongs to the phylum Glomeromycota (Remy et al. 1994; Schüßler et al. 2001). Arbuscular mycorrhizal (AM) fungi live in mutualistic associations with 80–90% of higher plants (Harley and Harley 1987). The features that make AM fungi suitable to promote phytoremediation of HM-polluted soils include their ubiquity, ability to provide nutrients to plants and support water uptake of host (Marschner 1997), enhance partner stress tolerance and affect metal transport (Gaur and Adholeya 2004; Göhre and Paszkowski 2006). The reports on the role of AMF infection on host metal uptake and transfer are contradictory (Leyval et al. 1997; Rahmanian et al. 2011), and therefore, both increases and decreases in metal concentration of mycorrhized plants are described (Karimi et al. 2011). While analysing the scientific impact of mycorrhiza on plant growth, a lot of aspects, for example, the chemical and physical properties of the contaminated soil (Killham and Firestone 1983; Wang and Chao 1992); the quality, quantity and availability of the polluting metal (El-Kherbawy et al. 1989; Guo et al. 1996); and the degree and the term of the load, the plant (Díaz et al. 1996; Kucey and Janzen 1987) and fungus species (del Val et al. 1999) and their ecotypes (Malcova et al. 2003; Wu et al. 2007), should be considered. Therefore, considering the relation between heavy metal uptake and relative plant growth parameters, two conceptual models were presented: (1) "enhanced uptake", which means increased HM uptake via mycorrhizosphere at low HM concentrations in soil, and (2) a reduced HM bioavailability via AM fungal "metal-binding" processes at high soil-HM levels (Audet and Charest 2007). These models broadly explain the interrelationship between plant growth, plant HM uptake and HM tolerance and consequently highlight the importance of AM symbiosis in buffering the soil environment for plants under such stress conditions. The AMF-enhanced method of phytoremediation can either be phytostabilization by which the entry of pollutant into food chain is inhibited or phytoextraction which removes the contaminant, depending on the concentration of the pollutant. Therefore, there are common and different aims for inoculation: reduction or rise of metal accumulation and uptake, augmentation of stress tolerance and biomass production. However, both technologies require the purpose-oriented selection of compatible fungus–plant partners.

11.3   Choosing Remedial Technology

The steps to prepare a site-specific, mycorrhiza-enhanced technology include the following:
1. Risk identification through the characterization of the polluted area
2. Risk assessment
3. Choice of phytoremediation technology according to the priorities
4. Development of adequate technology
   Selection of potential host plant
   Test and selection of infective and effective AMF strains
   Establishment of co-operating plant–fungi pairs
5. Monitoring of effectivity of the chosen technology
   Bioindicators and biomonitoring for human health risk (Fig. 11.1)

The priority of phytoremediation is determined mainly by the human health risk (EPA 2001). The human health risk assessment involves evaluating the effect of toxins, contaminants and other environmental hazards on human health. The aim of the operations is to reduce and minimize the risk with the chosen remediation technology. During the planning phase, the orientation, the actual and planned usage of the area, the degree and extent of the pollution and the protection of water should be considered. Other important aspects are the origin of the
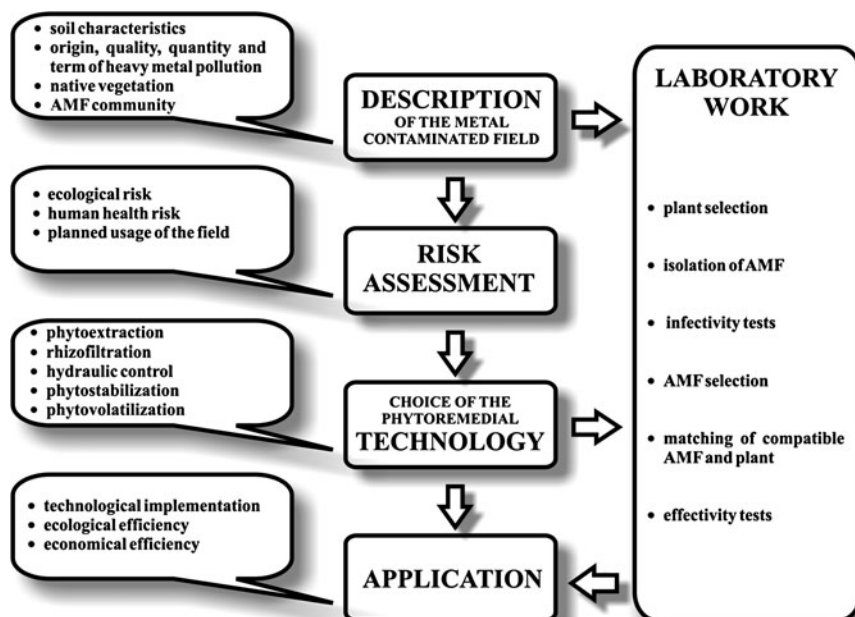
Fig. 11.1 Important considerations and investigations needed for the implementation of mycorrhiza-enhanced phytoremediation

pollution including duration, former conditions and usage, and the properties of pollutants, e.g. quality, quantity, volatility, chemical and biological stability and availability. Physical and chemical characteristics of the soil should also be examined. If there are alternative methods for risk prevention and reduction, the environmentally, ecologically and economically most efficient one should be preferred. The indigenous AMF population and vegetation are partially determined by the physical and chemical properties of the polluted soil, limiting the selection and the available technologies (Estún et al. 2002).

11.4 Selection of Host Plants

11.4.1 Metal Tolerance of Plants

The choice of the development of an AMF-optimized phytoremedial method lies in the selection of appropriate plant that could serve as an ideal host for AMF. For phytoextraction and phytostabilization technologies, the selected plant should be able to tolerate HM content of soil. Moreover, the toxic components should not be able to retard plant growth since high biomass yield is important especially for phytoextraction. The differences in metal tolerance of species or ecotypes are, however, determined genetically and manifested in the diversity of metal uptake, ionic and water transport, sequestration and binding ability of cell wall (Machnair 1993). There are three different categories of plants living in HM-polluted soil (Baker 1981; Leung et al. 2007): (1) excluding ones (excluders) that accumulate metals in root and prevent metal accumulation in shoots, (2) indicators where the metal concentration of organs varies in direct proportion to that of soil and (3) hyperaccumulators that concentrate the metal in their shoots over $1,000\,\mathrm{mg\,kg^{-1}}$ dry weight, a dose higher than that of the soil.

From the mycorrhizal point of view, although Glomeromycota includes few species, AMF are one of the most widely spread soil-borne fungi (Gerdemann and Nicolson 1963). Approximately 150 AMF species are described, but associations are formed with 200,000 plants including mosses, ferns, gymnosperms and angiosperms (Morton and Bentivenga 1994). The lack of host-specific symbiosis is advantageous for remedial plant selection. Establishment of purpose-oriented mycorrhiza is complicated by variability in host mycorrhizal dependence (MD), partner preference, the non-mycotroph plants and failures of infections (Table 11.1). Mycorrhizal dependence of the host plant is genetically controlled (Azcon and Ocampo 1981), but environmental conditions influence the response to AMF infection and colonization, causing differences in biomass production and uptake of nutrient and HM. A comparison of 250 plants revealed that MD of native species was higher than that of cultivated ones, and MD was inversely proportional to the measured parameters indicating the uptake capabilities (Tawaraya 2003).

Table 11.1 Types of heavy metal contaminated media and phytoremediation processes applied with commonly used mycotroph and non-mycotroph plants

Medium	Type of plants											
	Mycotroph plants							Non-mycotroph plants				
	Alfalfa	Sunflower	Grasses	Black locust	Hybrid poplar	Willow	Cottonwood	Alyssum	Thlaspi	Indian mustard	Water hyacinth	Pennycress
Soil	PV	PE	PS	PV	PE PS PV	PE PS		PE	PE	PE PS PV		PE
Sediment	PV	PE	PS	PV	PE PS PV	PE PS		PE	PE	PE PS PV		PE
Groundwater	RF				HC	HC	HC			RF	RF	

Modified table of EPA (2001). *HC* hydraulic control, *PD* phytodegradation, *PE* phytoextraction, *PS* phytostabilization, *PV* phytovolatilization, *RF* rhizofiltration

11.4.2 Non-mycotroph or Unconcerned Hyperaccumulating Species

Within terrestrial plants, about 400 species of *Asteraceae*, *Brassicaceae*, *Cariophyllaceae*, *Cyperaceae*, *Cunoniaceae*, *Fabaceae*, *Flacourtiaceae*, *Lamiaceae*, *Poaceae*, *Violaceae* and *Euphorbiaceae* can hyperaccumulate (Brooks 1998). Among these families, crucifers top the list with 87 species including 11 genera. Although these wild hyperaccumulator plants are capable of phytoextraction, these species are generally not or slightly infected with AMF; in latter case, arbuscules are not formed (Newman and Reddell 1987). These plants must have a surviving mechanism to achieve the metal tolerance, which is mostly escalated by AMF in other cases (Leyval et al. 1997; Salt et al. 1995). In the last few years, more and more authors report that plants classified as non-mycotroph can be infected and may establish functional symbiosis in extreme conditions (Füzy et al. 2008; Regvar et al. 2003; Vogel-Mikus et al. 2005).

Violaceae was enrolled as an unconcerned family, but in HM-polluted soil, the AMF colonization of zinc accumulator violet subspecies (*Viola calaminaria* ssp. *westfalica*, *V. calaminaria* ssp. *calaminare*) was observed (Hildebrandt et al. 1999). In pot experiment, a *Glomus intraradices* strain isolated from the rhizosphere of zinc violet could enhance the metal tolerance of both dicots and monocots (Kaldorf et al. 1999). In contrast, a *Glomus* sp. isolated from under zinc violet increased the cadmium and zinc uptake of clover root (Tonin et al. 2001). AMF was also found in the root of *Thlaspi praecox* Wulfen (Regvar et al. 2003; Vogel-Mikus et al. 2005). The cadmium and zinc accumulator pennycress had been thought of as a non-mycotroph; the special feature of the discovered symbiosis was the shift of Arum and Paris type of colonization depending on the HM concentration. To apply inoculated pennycress and other unconcerned hyperaccumulators for phytoextraction, the difficult signalling and controlling system of symbiotic partners should be understood (Khan 2005). The usage of hyperaccumulating plants is, however, restricted by the fact that both concentration limit and metal uptake by species are metal specific.

11.4.3 Choosing Indigenous or Exogenous (Cultivated) Plant Species

Remediation in general is aimed at cleaning the polluted soil, reconditioning soil functions and reducing the environmental risk. On the other hand, restoration means rebuilding the ecosystem with its functions. The fundamental objective of combined or complex remediation is, therefore, to completely reduce the environmental risks caused by HM pollution with the establishment of a nearly native, self-sustaining ecosystem. This method is inexpensive and has some human health advantages, for example, the appearance of invasive, allergic weeds (*Ambrosia* sp., *Solidago* spp.) spreading at the beginning of succession is usually suppressed. For the complex method, generalist plant species are selected from the native, climax community by considering their metal and disturbance tolerance and uptake capabilities (Simon and Biró 2005; Takács et al. 2008; Turnau et al. 2008).

Resettlement and survival of indigenous plants, however, can be promoted by the co-application of AMF strains that are compatible with most of the native plant species and have no vantage over aboriginal AMF (Ryszka and Turnau 2007). The possible methods of phytoremediation are heavily influenced by the composition of the native vegetation and the survival strategy of plants (Regvar et al. 2006). Because of the easier management and larger biomass production, it is common to use cultivated species for phytoremediation, sometimes along with indigenous plants in pre-, inter- or co-cropping systems. To select and test AMF strains in order to investigate their metal uptake capacity, experiments are usually conducted with agricultural species. For example, sunflower (*Helianthus annuus*) is often applied to remove arsenic, chromium, uranium and other radioactive materials (Davies et al. 2002; Ultra et al. 2007). Cereals, primarily maize (Liao et al. 2003; Malcova et al. 2003), sorghum (del Val et al. 1999) and wheat (Tullio et al. 2003), and some less metal tolerant cultivated legumes like bean, alfalfa and clover (Heggo et al. 1990; Joner and Leyval 1997; Vörös et al. 1998; Zhu et al. 2001) have also been tested.

11.4.4 Advantages and Disadvantages of Trees

Phytoremediation technologies that involve the use of trees are less expensive, and extended and robust root system penetrates the soil by several metres, so large area can be cleaned. In addition, it is advantageous that the above-ground biomass can be harvested annually and the tree sprouts again, without disturbing the soil. The produced biomass could be a source for bioenergy. Some of the commonly used trees are willow and poplar species or their clones. Of these, willow is applied for the extraction of cadmium, zinc and copper (Mathe-Gaspar et al. 2005; Vysloužilová et al. 2003), whereas poplar has been used to remove lead (Takács et al. 2005, 2008). The space demand in a climate chamber, the change of mycorrhiza dependence by age, the coexistence of endo- and ectomycorrhiza and the vegetative propagation (cloning) together all make the directed inoculation of the arboreal plants much more difficult compared to herbs. AM fungi predominate in the early stages of a wood's life, where ectomycorrhizal fungi also infect the host. In this case, understanding the colonization process/efficiency and mycorrhizal effectivity is important to achieve optimum results.

11.5   Selecting Infective and Effective AM Fungi

11.5.1 The Effect of HMs on the Abundance and Vitality of AMF

Attention in recent years has been paid onto understanding the diversity, infectivity, HM adaptation and tolerance of AMF in order to better explore the possibility of effectively using AM fungi in bioremediation and, consequently, restoration of polluted soils. Most of the data suggest that heavy metal load of soil retards the

infection by AMF (Leyval et al. 1995), and therefore, root colonization by AMF has been found to decrease in the presence of HMs (Weissenhorn and Leyval 1996; Hoflich and Metz 1997). Metal pollution can even totally block the formation of plant–AMF symbiosis (Koomen et al. 1990; McGee 1987), sporulation (Liao et al. 2003; Tullio et al. 2003), in vitro germination of spores (Weissenhorn and Leyval 1996) and the spreading of hyphae (Pawlowska and Charvat 2004). Several authors have shown that AMF can adapt to a long-term metal pollution and HM-tolerant AMF strains can be selected by contamination (Weissenhorn et al. 1995; Weissenhorn and Leyval 1995; Del Val et al. 1999). In phytoremediation, AMF selection is determined by the kind of technology to be applied and the plant–fungi relationship. For example, in phytoextraction, hydraulic control and rhizofiltration, the strain to be developed should promote metal removal and, as far as possible, biomass production. In phytostabilization, AMF inoculation should reduce the metal content of the host, which can be obtained by controlling both metal uptake and biomass yield (Alten et al. 2002).

11.5.2 Investigating the AMF Community of the Polluted Area

Pre- and post-remediation investigations on the composition and the infectivity of the indigenous AMF community can provide informations to understand the sustainability and functionality of phytoremediation systems (Dodd and Thompson 1994; Leyval et al. 1995). AMF inoculations are important especially in metal-contaminated coal and ore strip mines, where both vegetation and microbial community are destroyed. Over metal aggregation the structure of the soil changes to compact and water permeability is decreased. In the spoil there are minimal available nutrients. In such extreme conditions, the presence of resistant, effective AMF strain can contribute to supply the minimal needs of some plants, so AMF can make plants survive (Leung et al. 2007).

For site-specific phytoremediation, indigenous or native species should be identified and applied. To this end, it is important to investigate extensively the AMF community inhabiting long-term polluted sites. In a phytoremediation system, the strains adapted to the contamination are possibly more efficient than non-adapted ones. For example, in 1991, a long-term heavy metal load field experiment (Kádár 1995) was set up, where small areas were polluted with three concentrations (30, 90 and 270 mg metal kg^{-1} dry soil) of 13 selected metals. High concentrations and long-term HM loads resulted in distinctive changes in diversity while sensitive species disappeared. However, some tolerant species survived and adapted to the contaminated environment (Takács et al. 2000). In the seventh and eighth year after metal application, six AMF species, namely *Glomus claroideum, G. constrictum, G. mosseae, G. microcarpum, G. sinuosa* (*Sclerocystis sinuosa*) and *Glomus* sp., were found in the ploughed layer of the cadmium-, nickel- and zinc-treated and control plots. Two control plots included a cultivated and fertilized one which represented the agricultural conditions, while an uncultivated one was allocated in the natural ecosystem nearby. High and intensive agronomic input reduced the
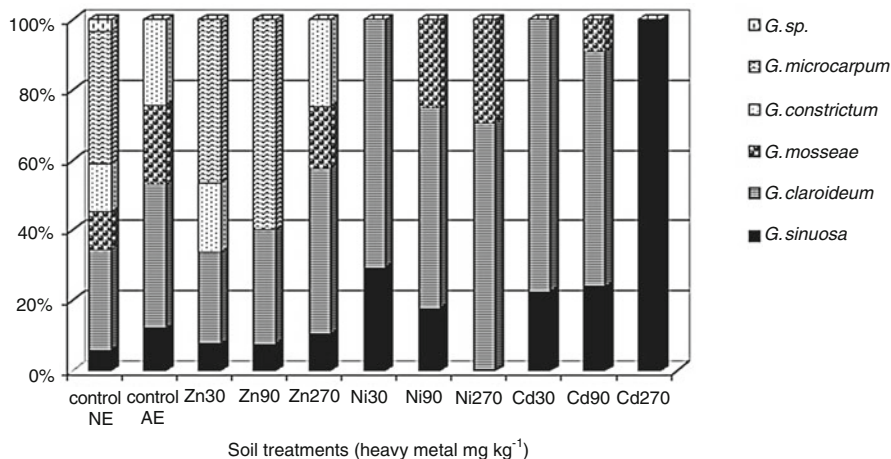
Fig. 11.2 Spore frequency of different AMF species collected from undisturbed (C_{NE}), disturbed (C_{AE}) and cadmium-, nickel- and zinc-contaminated soils (30, 90, 270 mg kg^{-1})

number of fungal species compared to natural ecosystem. A further decline in AMF diversity and abundance was observed that were influenced by the quality and concentration of the HM (Fig. 11.2). Cadmium was found as the strongest inhibitor, at the highest Cd dose (270 mg kg^{-1}) only *G. sinuosa* could survive. Two species, *G. mosseae* and *G. claroideum*, were detected in the soil contaminated with nickel to the highest degree. Zinc had the least impact, and each of *G. sinuosa, G. claroideum, G. mosseae* and *G. constrictum* tolerated the highest zinc level. Among these, *G. claroideum* and *G. sinuosa* were the most resistant species, and their spores were abundant in all but one treatment. *G. mosseae, G. sinuosa* and *G. claroideum* spores were also isolated, and the derived strains were HM tolerant (Vörös and Takács 2001). The outdoor phytoremedial application of the inocula was successful against different contaminants (Takács et al. 2008). *G. mosseae, G. claroideum* and *G. microcarpum* have also been isolated from several heavy metal polluted soils (Weissenhorn et al. 1994; Vivas et al. 2003; Ortega-Larrocea et al. 2007; Zarei et al. 2010). *Glomus microcarpum* spores were collected from zinc-, copper-, lead-, nickel- and cadmium-contaminated soils (Sambandan et al. 1992). Furthermore, *G. claroideum* spores were found in zinc-, copper-, lead-, nickel-, cadmium- and arsenic-contaminated habitats (Del Val et al. 1999; Turnau et al. 2001). The HM tolerance of *G. claroideum* originated from a field contaminated with sewage sludge is enhanced compared to strains in native soil (Del Val et al. 1999). In high-input agricultural practices, soil ploughing, fertilization and fungicide application, however, can reduce the infectivity and efficiency of AMF on host growth and nutrient uptake by inhibiting the functions of the extraradical hyphal network (Jansa et al. 2002). For example, mycorrhizal inoculum potential, spore abundance, diversity and structure of AMF communities have been affected by long-term tillage and HM pollution (Ortega-Larrocea et al. 2007).

The mechanism of metal tolerance is complex and not fully understood. Most of the toxic metal ions are bound to coordination compounds and can partially or completely be sequestered to the soil or immobilized in solid phase (Joner et al. 2000; Schutzendubel and Polle 2002). Immobilized metals can be deposited in cell wall (Göhre and Paszkowski 2006; Gonzalez-Guerrero et al. 2008) or in vacuoles. The stages of the symbiosis can show different sensitivity to metal pollution. As an example, sporulation was more sensitive to metal exposure than symbiotic mycelium expansion (Pawlowska and Charvat 2004). In my opinion, the type of AMF sporulation such as the development of peridium around spores has an important role in protection against mechanical and chemical disturbances in soils. Therefore, the kind of sporulation and the morphology of the spores influence the metal tolerance of AMF species. Spores of both *G. sinuosa* and *G. mosseae* are covered with a dense hypha layer to serve as a mechanical barrier, but filtration against HMs can also be supposed. With the peridium around the sporocarp or single spore, *G. sinuosa* and *G. mosseae* have advantage for spreading in disturbed soils (Takács et al. 2000). The taxonomical position manifesting in different functional and morphological features can determine the utility of AMF species for phytoremediation (Morton and Bentivenga 1994; Hart and Reader 2002; Liao et al. 2003). Both reported data and morphological signs promote the selection of species from the indigenous AMF community, shortening the period of inoculum development.

11.6 Effect of HM-Adapted and HM Non-adapted AMF on Host Tolerance: A Comparative Study

The adaptation of indigenous AM fungi was investigated in case of long-term cadmium, nickel and zinc application (Takács et al. 2001). Within the same cultivated area, differently contaminated and pure, undisturbed soil samples were collected and were later treated with HMs corresponding to the level of long-term contamination. In pot experiment, ryegrass (*Lolium perenne* L.*)* was grown to check the colonization parameters and the effect of AMF on metal uptake. In the root of ryegrass infected with adapted fungi, the extent of arbuscules increased with the increasing metal load. Fungi from pure soil, however, formed comparatively lower number of arbuscules at the same metal concentration (Fig. 11.3).

Investigations on metal transfer from soil to plant promote the understanding of heavy metal uptake process of mycorrhiza-infected plants (Kabata-Pendidas 2004; Redon et al. 2009) and the estimation of human health risk caused by the pollutant (Anton and Máthé-Gáspár 2005). Regarding the amount of accumulated metals, roots and shoots showed a variable response. In shoots, the order of metal concentrations was zinc, nickel and cadmium. On the contrary, roots accumulated cadmium at the highest level. As our experiments have proven, the role of the metal in plant physiology should be considered beyond its concentration. Zinc and nickel are essential elements for the physiological and biochemical functions of higher plants; it can explain the differences in metal uptake even at high level of metals in the soil. However, metals that are natural components of plants usually harm humans less.
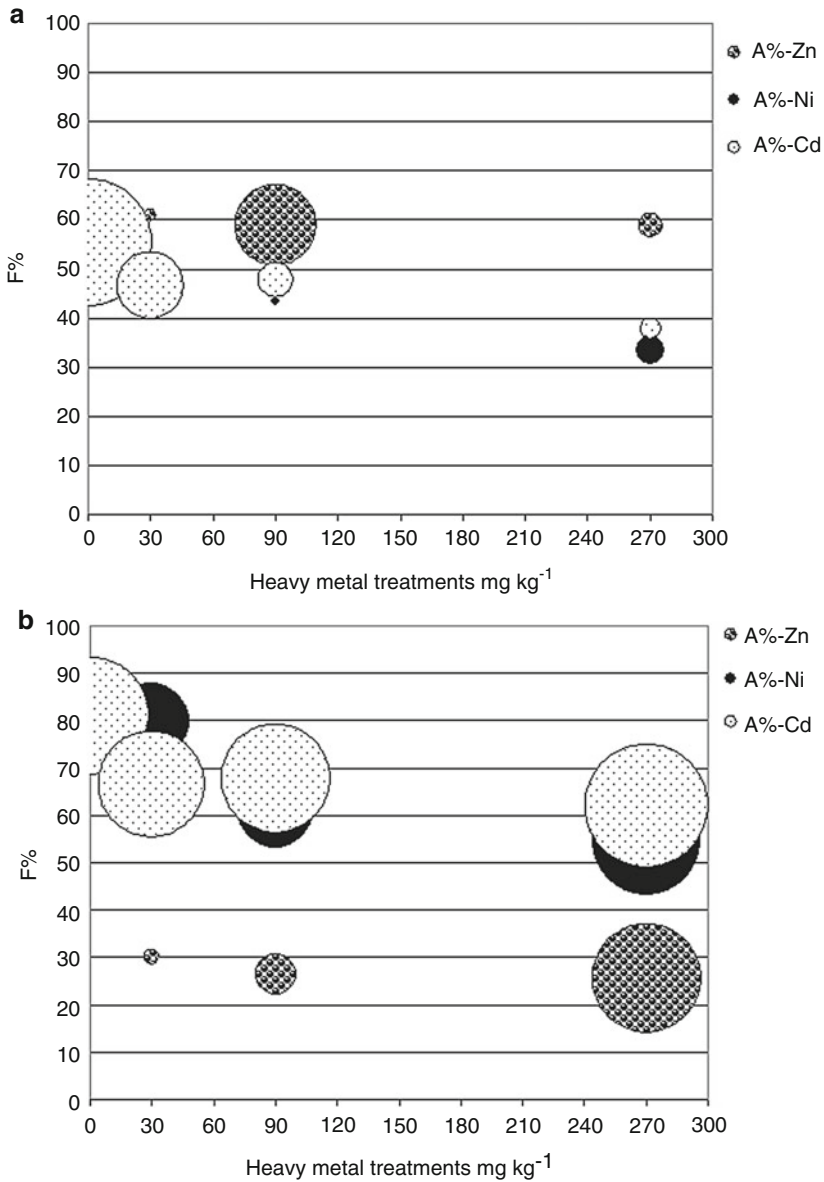
Fig. 11.3 Frequency of colonization (F%) with metal non-adapted (**a**) and metal-adapted (**b**) AMF in ryegrass at different exposure rates of zinc, nickel and cadmium. Bubble size reflects the arbuscular richness (A%)

Table 11.2 Soil–plant transfer (BCF = $c_{shoot}/c_{soil\ available}$) of the metals in mycorrhizal ryegrasses

Element		Originally applied metal rates (mg metal kg^{-1} dry soil)			
		0	30	90	270
BCF_{Cd}	a	0.77	0.23	0.14	0.04
	b	0.82	0.25	0.22	0.13
BCF_{Ni}	a	1.27	1,57	1.42	1.74
	b	0.10	0.65	0.60	0.58
BCF_{Zn}	a	27.3	7.07	5.70	3.49
	b	12.0	2.70	1.91	1.92

*BCF*a of metals in ryegrass infected with metal-adapted AMF
*BCF*b of metals in ryegrass infected with metal non-adapted AMF
Data are mean of three replicates

Previously absolute concentrations could be compared because of the similar doses of the different polluting metals, but generally, the bioconcentration factor (BCF), which means the concentration of a material in the plant relative to that in the soil, characterizes the ability to accumulate HMs better. For example, AMF of HM-polluted soil could decrease metal transport from cadmium- and nickel-polluted soil to the host plants more intensively than fungi of undisturbed soil as it is presented by the different BCFs (Table 11.2). In case of fungi inhabiting HM-polluted soil, the ability to decrease metal uptake is due to both adaptation and effectivity of AMF; however, it is not always related to infectivity of AMF. A good colonization of indigenous AMF could be accompanied by inefficiency (Rydlova 1998), and there could be no detectable differences between the germination of HM-adapted and HM non-adapted spores (Vosatka et al. 1998). Even though there are inconsistencies in the reported results, it is reported that AMF strains stressed by long-term HM pollution have enhanced tolerance to HM and infectivity to host plants, compared to fungi inhabiting undisturbed areas (Leyval et al. 1997). According to the literature, the application of mycorrhizal fungi reduced the yield of ryegrass (Fitter 1977; Yu et al. 2004).

11.7 The Impact of Intra- and Interspecific Variability of AMF on Metal Uptake

Genetic diversity within species is common, but in case of AMF, it is enhanced by the coenocytic hyphae and spore formation (Bever et al. 2008; Pawlowska and Taylor 2004). The morphology, the colonization properties of AMF, the functionality and efficiency of the symbiosis all show high intra- and interspecific variability (Jakobsen et al. 1992; Munkvold et al. 2004; Van der Heijden et al. 2004; Cavagnaro et al. 2005). The structure of the natural vegetation is highly influenced by the diversity and the composition of natural AMF communities (Vandenkoornhuyse et al. 2002; Klironomos 2003). Although AMF–host relationship is not strictly specific, diversity and functionality are unique (Helgason et al. 2002; Takács et al. 2005). In a comprehensive investigation, cultures of four AMF species (*Glomus mosseae*, *G. claroideum*, *G. caledonium* and *G. geosporum*) derived from 24 sites showed

Fig. 11.4 (a, b)
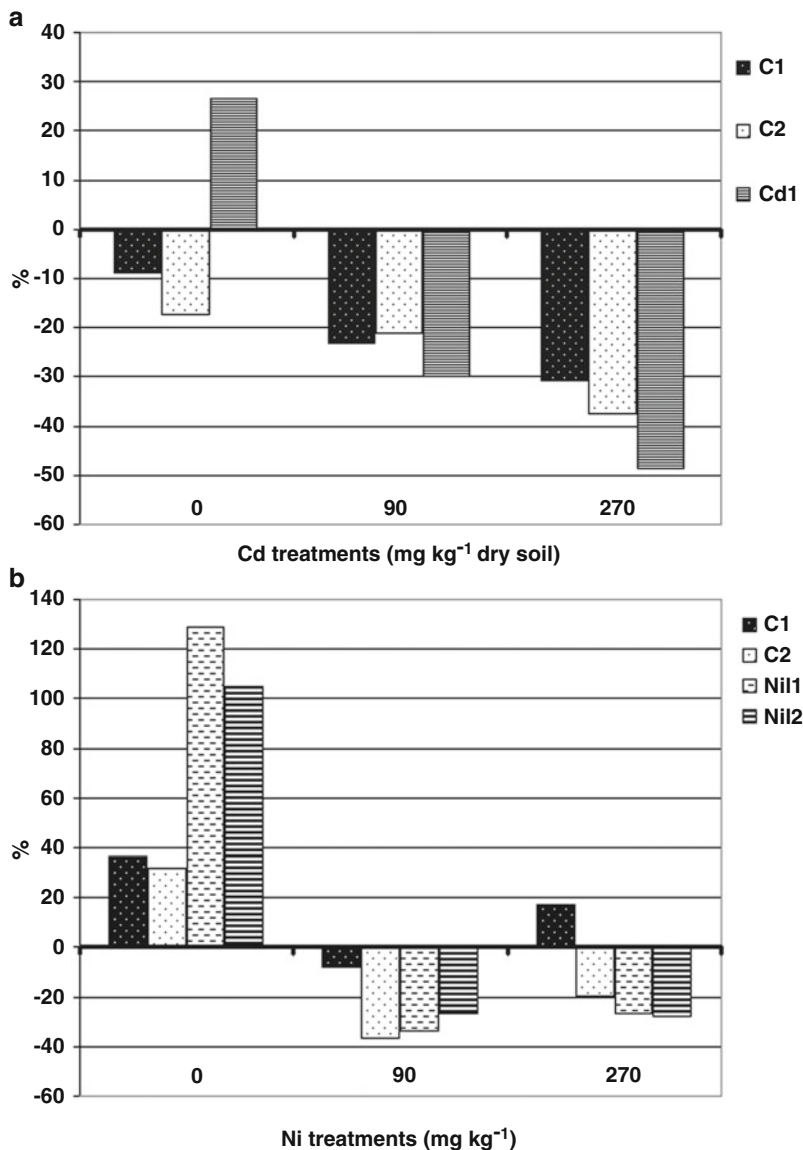
functional variation among both species and strains, when they were tested for their effect on the growth and P uptake by cucumber (Munkvold et al. 2004). In a manner similar to the differences occurring in the response of the plant to the colonization, the effect of AMF strains on yield or nutrient, water and HM uptake by host plants also varies (Fig. 11.4). Apart from the environmental conditions, the phytoremedial utility of AMF is also influenced by their origin (Leyval et al. 1997; Vörös and Takács 2001)
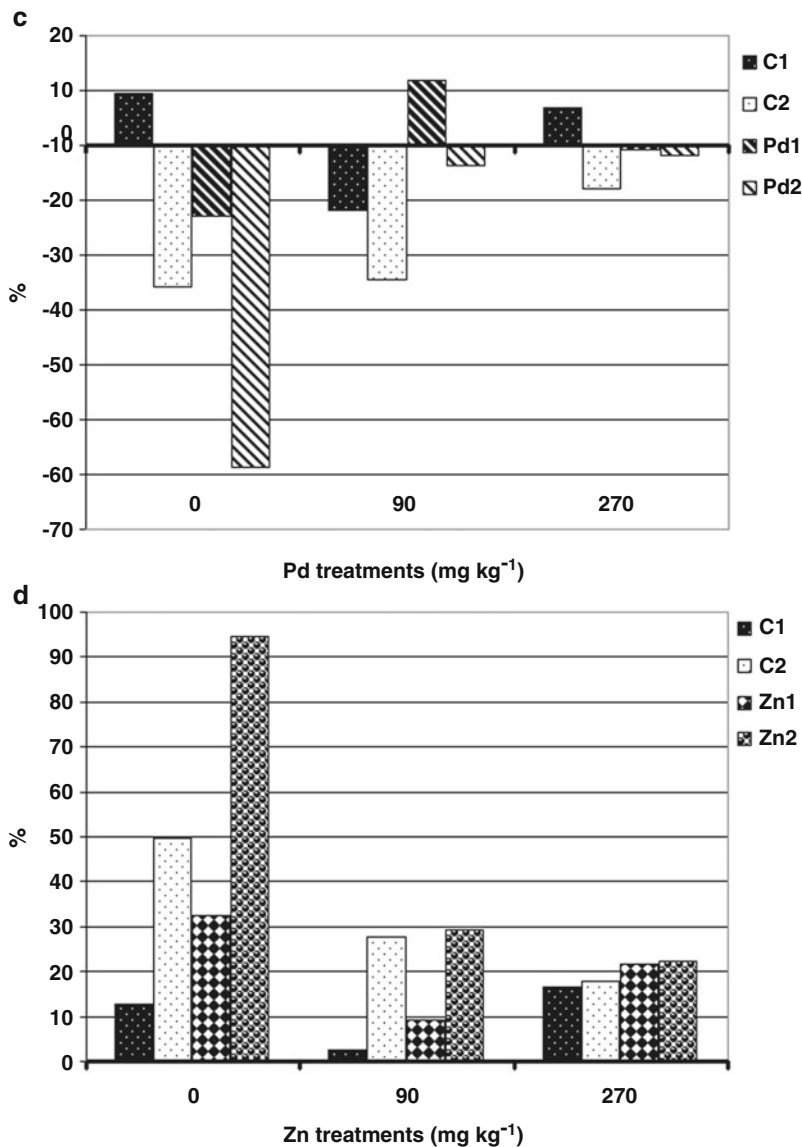
Fig. 11.4 Changes in the amount of cadmium (**a**), nickel (**b**), lead (**c**) and zinc (**d**) accumulated in cucumber leaves; values are expressed in percentage of the non-mycorrhizal control. Heavy metal accumulation in cucumber were investigated in soils treated with Cd, Ni, Zn and Pb at 0, 90, 270 mg kg^{-1} doses of selected HMs. AMF used as inoculant were nine *Glomus* sp., from which two ones (C1-2) were non-adapted control. The other strains (Cd1;Ni1-2; Zn1-2; Pb1-2) are inhabitants of different polluted soils. Data are mean of 3 replicates
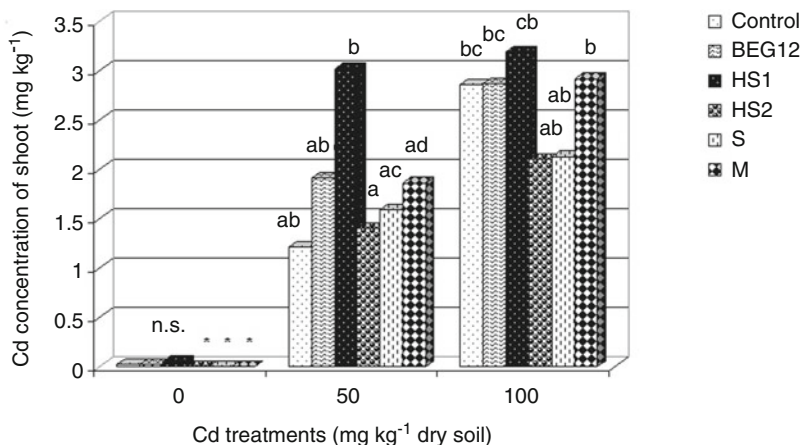
Fig. 11.5 The impact of intraspecific variability of *Glomus mosseae* strains on cadmium concentration (mg kg^{-1}) in shoots of white clover grown in soils treated with 0, 50 and 100 mg Cd kg^{-1} BEG12: obtained from the European Bank of Glomeromycota; S: spores isolated from salt-affected soil; M: spores isolated from cadmium polluted soil; HS: spores isolated from humus sandy soil. Different lower case letters indicate significant differences between AMF inoculated plants at P < 0.05. Data are mean of 3 replicates. *Concentrations are below detection limit (Cd: 0.02 mg kg^{-1})

and the metal transport capacity of the plant (Tonin et al. 2001). In our experiment, white clovers were inoculated with five strains of *G. mosseae* originating from different undistorted, HM-polluted and saline sites. Cadmium uptake of host plants varied depending on the original habitat of the fungi, but HM- or salt-adapted strains were not always significantly superior in efficiency compared to native ones (Biró and Takács 2007) (Fig. 11.5). The functional difference among AMF species and strains was investigated further, and it was observed that simultaneous treatment with several efficient strains having similar influence on metal uptake could be more prosperous (Khan et al. 2008) or can produce unexpected results (Gosling et al. 2008). An AMF community that is rich in species could improve the productivity of plants better than the one with low species number due to the easier meeting of adequate partners (Maherali and Klironomos 2007). It is, however, difficult to choose the adequate HM-tolerant fungi and the possible supplemental non-adapted strains for the effective combined inoculum. Therefore, use of one-step restoration with multiple plant species and tolerant fungi should be considered (Bever et al. 2001).

11.8 Sustaining Heavy Metal Tolerance and Inducing Artificial Adaptation

There are two ways by which mycorrhizal inocula could be employed in remediation technologies: (1) either indigenous AMF can be mixed with exogenous, HM-tolerant ones or (2) aboriginal fungi should be made adapted to the HM pollution in vitro.

Table 11.3 Changes in the amount of metal accumulated in *Populus nigra* leaf in percentage correlated to the control

Treatments	Heavy metals (%)				
	Cd	Mn	Ni	Pb	Zn
Glomus mosseae-P1	64.9	56.6	39.9	247	2.17
Glomus mosseae-D1	18.2	66.5	−14.0	−55.9	30.76
Glomus mosseae-P2	53.4	79.9	15.7	−6.52	40.23
Glomus mosseae-D2	30.5	−22.2	−56.4	−100	−39.64

Heavy metal accumulation in poplar trees was investigated in soils treated with five heavy metals: Cd 20 mg kg^{-1} (3 CdSO$_4$ 8 H$_2$O), Mn 20 mg kg^{-1} (MnSO$_4$ 7H$_2$O), Ni 20 mg kg^{-1} (NiSO$_4$ 7H$_2$O), Pb 10 mg kg^{-1} (Pb(NO$_3$)$_2$) and Zn 50 mg kg^{-1} (ZnSO$_4$ 7H$_2$O). AMF used as inoculant included four *Glomus mosseae*, two parents (P1-2) and two descendants (D1-2). The *G. mosseae* P1 were isolated from Cd loaded soil in a long-term field experiment. *G. mosseae* P2 was isolated from an undisturbed sandy soil in Hungary. The descendant substrains D1 and D2 of *G. mosseae* (P1, P2) strains were propagated under heavy metal stress before application to stabilize and adapt their characteristics. Data are mean of nine replicates

There are also opinions that the acquired feature of metal tolerance by AMF may be lost during repeated propagations (Sudova et al. 2007). So, in order to maintain the infectivity, it is expedient to reproduce the inoculum under stress conditions similar to those matching with field environment. The in vitro simulation of outdoor conditions to evolve and sustain phytoremedial effectivity is often referred to as "directed inoculum production process" (DIPP) (Feldmann and Grotkass 2002). Comparing to routine propagation methods, by the continuous presence of the stressor, DIPP provides a better chance to develop or sustain strains that may become successful in remediation. Apart from the adaptation method, to develop HM tolerance in a strain, the strain should be grown with the optimal dose of the selective metal. The term of the adaptation also varies with AMF species and metals. For example, the tolerance of a *Glomus claroideum* strain of the three isolated species was proven a year after pollution of soils with sewage sludge (Del Val et al. 1999). As metal tolerance of AM fungi native to contaminated fields was passed on during propagation in unpolluted soil (Leyval et al. 1995), adaptation is rather a genomial change than an acquired characteristic. By infecting *Anagallis arvensis* host with subsequent descendants of a single spore *Glomus* sp. strain, only slight differences were found in mycorrhizal effect on the biomass production between the generations (Feldmann and Grotkass 2002). Applying soil pH as selective agent in DIPP can change the functional composition of an AMF population. In our experiment, the HM adaptation capability of AMF was examined. Two monosporic *G. mosseae* strains derived from different ecosystems were grown in soil containing 100 mg kg^{-1} cadmium for 5 months (Takács et al. 2008). Micropropagated *Populus nigra* cuttings were inoculated with parent (P1, P2) and descendant (D1, D2) strains during acclimatization (Table 11.3). There were differences in root colonization properties of the strains. The commemorative propagation stabilized the features of the P1 strain originated from cadmium-polluted soil. Depending on the metal and the strain, the cadmium, manganese, nickel, lead and zinc accumulation capacities of poplar were increased by 2–247% after the inoculation.

Conclusion

There is no doubt about the importance of AM fungi in phytoremediation systems. The interaction of the host plants with the AM fungi, however, depends on the genotype of the partners and on several environmental factors (Johnson et al. 1997). Moreover, the comparison of reported data is a major problem since the degree of HM contamination in soil is generally defined by the total metal amount while toxicity hangs on the biologically available concentration. And hence, it becomes difficult to assess the toxicity. Therefore, issues of effective AMF-enhanced phytoremediation should be explored or refined. For example, the physiological effects of HM on symbiosis, assessment of lethal concentration of HM for each of the plant, the fungus and the symbiosis in addition to the comparative analysis of genetical diversity along the gradient of pollution should be studied. The impact of seasonal variation on plant, fungus and symbiosis should also be investigated thoroughly because the effects of the AM fungi may vary periodically. The success of inoculum thus depends on the metal tolerance ability of AM fungi, the maintainability of the acquired feature, the possibility of in vitro adaptation of the strain to the stress and the understanding of taxonomical, morphological and sporulation features of mycorrhizal fungi. The preconditions for detailed analysis of fungal tolerance include the development and complex application of test methods for metal toxicity and the comprehensive evaluation of ecological, taxonomical, environmental monitoring, in vitro and outdoor ecotoxicological data. To achieve optimum success in mycorrhiza-mediated phytoremediation, the application of suitable and host-specific inoculum and interdisciplinary assessment of mycorrhizosphere are required. Therefore, based on the literature available, it can be suggested that inoculation of plants with indigenous and presumably stress-adapted AM fungi could serve as a potential biotechnological tool for achieving greater success in restoring destructed/degraded lands with minimal input.

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Heavy Metal Resistance in Plants: A Putative Role of Endophytic Bacteria

12

Iryna Zaets and Natalia Kozyrovska

Abstract

Heavy metals and metalloids have become one of the major environmental concerns which pose a serious threat to plants and animal health. In this context, endophytic bacteria could play an important role in understanding the uptake mechanism of heavy metal ions and providing immunity to plant against metal toxicity. The defensive effects of certain elements in plants are known, but the role of endophytes in providing protection to plants has poorly been investigated. Endophytic bacteria, originating from hyperaccumulator plants, exhibit a comparatively higher level of resistance to heavy metals than the soil and the rhizosphere bacteria. Among bacteria, *Methylobacterium* spp., as well as the representatives of Gram-positive bacteria, are the most widespread bacterial species in both the hyperaccumulator endosphere and endorhizae. The endophytic microbial populations enhance the resistance capacity of the host plants, which, however, depends on the structure and activity of the community. Moreover, endophytic bacteria including those of legume endophytes are considered a promising biological material for improving the efficiency of phytoremediation and, consequently, growing of clean and safe crops including legumes in metal polluted soils. The recent developments in the putative mechanisms by which endophytic microorganisms affect the plant resistance to heavy metals and how they could affect phytoextraction of metals from contaminated soils are highlighted.

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12.1   Introduction

Heavy metals (HM) in general are known as the most toxic pollutants of the environment. Some metals, such as Ca, Cu, Mg, Mn, Zn, Mo, and Fe, are essential nutrients for plants, while others like Cd, Co, Cr, Hg, Pb, and Ni are not. Irrespective of whether they are essential or not, heavy metals at elevated levels are lethal. Furthermore, most of the HM have a low mobility in soil and are not easily absorbed by plant roots (Garbisu and Alkorta 2001; Chen et al. 2004). Soil- and plant-associated microbes on the other hand are able to leach and immobilize heavy metals in soils. The resistance to noxious metals among bacterial species is known (Trajanovska et al. 1997). For this, several bacterial species possess genes responsible for resistance to HM and have evolved a variety of mechanisms to reduce HM stress (Alonso et al. 2000; Van Houdt et al. 2009; Khan et al. 2009). These mechanisms include the complex formation and sequestration of HM, reduction of a metal to less toxic species, induction of the oxidative stress response, a reduced membrane permeability, and direct removal of metals (Nies 1999; Tremaroli et al. 2009; Prévéral et al. 2009; Diels et al. 2009). There is also evidence which suggests that many species of endophytes are resistant to high concentrations of HM (Barzanti et al. 2007; Sheng et al. 2008; Kuffner et al. 2010). And therefore, bacterial strains isolated from inner tissues of many plants including legumes and capable of facilitating plants growth and reducing/detoxifying metal toxicity are urgently required because the plant–endophyte symbiotic system is considered a promising tool in increasing the efficiency of phytoremediation (Rajkumar et al. 2009). Considering this as a basis, Luo et al. (2011a, b) isolated endophytic bacterium *Serratia* sp. LRE07 from cadmium hyperaccumulator *Solanum nigrum* L. Importantly, LRE07 was resistant to the toxic effects of heavy metals, solubilized mineral phosphate (P), and produced indoleacetic acid (IAA) and siderophores. In addition, strain LRE07 bound over 65% of cadmium and 35% of zinc in its growing cells from single metal solutions 72 h after inoculation. Also, strain LRE07 removed sufficiently the Cd and Zn, when these metals were present in combination, indicating that the endophyte had explicit and amazing heavy metal abatement potentials. In a similar study, Wang et al. (2006) reported a total of 10^4–10^6 cells/g of fresh epidermis in the tissue of *Conzattia multiflora*, a leguminous tree grown in Mexico and Guatemala. The isolated bacteria were Gram-negative, facultative anaerobic rods, and formed yellow or colorless colonies. Later on, they were recognized as endophytes following inoculation tests and some of them could significantly promote the growth of *Conzattia* seedlings. Using PCR-based RFLP, they were found to belong to the genera *Pantoea*, *Erwinia*, *Salmonella*, *Enterobacter*, *Citrobacter,* and *Klebsiella* by the phylogenetic analysis of 16S rRNA genes.

In other study, Boyd and Martens (1992) proposed an "elemental defense hypothesis" which suggested that the plant protection against herbivores or pathogens can be provided by HM accumulated within hyperaccumulator plants, while Fones et al. (2010) reported a direct defensive effect of HM against plant pathogens. In addition, HM also induce a systemic acquired resistance (SAR) in

plants via a salicylic acid (SA)-dependent signal transduction pathway analogous to pathogenic necrotrophic bacteria (Yang et al. 2009). The high content of HM induces the synthesis of SA and other appropriate plant metabolites that finally cause SAR and the resistance to pathogens. In this context, the role of endophytes in plant defense against HM should be considered. In spite of scarce information on the development of the defensive mechanisms by plant via endophytes, it is known that some endophytic bacteria have the potential to activate both a basal MAMP-triggered immunity and inducible plant defense systems by interacting differently with variable host plants (Iniguez et al. 2005; Conn et al. 2008; Ardanov et al. 2011). There are other putative ways by which endophytes can alleviate the HM toxicity caused to plants. For example, through direct mechanism, endophytes reduce the availability and mobility of chemical elements by chelation, binding them with siderophores, the competitive acquisitions of less toxic elements. Also, endophytes can transform the oxyanions to nontoxic element forms and detoxify the metal by forming a complex with SH-groups (Pages et al. 2008). Bacteria also reduce the impact of HM indirectly by the secretion of biologically active substances, such as plant growth-stimulating hormones. The beneficial effects on the plant growth in the presence of HM have been attributed to endophytes and may include an osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals, and the alteration of the nitrogen accumulation and metabolism (Compant et al. 2005).

12.2   Endophytic Bacteria: Tolerance to Heavy Metals

Endophytic bacteria are the bacteria that reside within the living tissue of the host plants at least during a part of their lifetime without harming it (Wilson 1995). They are ubiquitous in most plant species and actively colonize the tissues and remain inside the plant latently until activated by the environmental stressors (Podolich et al. 2007; Lian et al. 2008). Endophytic bacteria have been isolated from various plants including legumes and tested for their ability to facilitate legume growth and to reduce heavy-metal toxicity. For example, a total of 31 endophytic bacterial species belonging to 14 different genera, recovered from the foliage (*Pantoea agglomerans*, 60%), tap roots (*Agrobacterium rhizogenes*, 49%), and nodules (*Rhizobium leguminosarum* bv phaseoli and *R. Loti*, 27% each) of red clover plants (*Trifolium pratense* L.), were used to assess their effects both alone and in combination with *Rhizobium* spp. on the growth and development of red clover seedlings (Sturz et al. 1997). Other than rhizobia, 12 endophytic bacterial species formed nodules on the root systems of clover plants. When grown on nonselective media, only 9% of total bacterial species were recognized as *R. leguminosarum* bv *trifolii* in the root nodule. Furthermore, upon inoculation, nodule bacteria enhanced the growth of red clover significantly when applied as mixture with *R. leguminosarum* bv *trifolii* compared to its sole application. The legume endophytes *Bacillus megaterium*, *Bordetella avium,* and *Curtobacterium luteum* constantly increased the growth used either alone or as mixed inoculation with *R. leguminosarum* bv *trifolii*. Nodulation was further

improved when *Bacillus insolitus*, *B. brevis,* or *A. rhizogenes* A was used with *R. leguminosarum* bv *trifolii*. Though, sole applications of *Rhizobium* species always led to the depression of clover growth, but mixtures of *R. leguminosarum* bv *trifolii* and *R. leguminosarum* bv *phaseoli* resulted in growth increment. Similarly, endophytic bacteria isolated from surface-sterilized stems, roots, and nodules of wild and cultivated soybean (*Glycine max*) varieties were motile and released IAA, cellulose (70%), and pectinase (33%), and a few isolates were resistant to antibiotics (Str_{100}), formed capsules, and produced fluorescent pigments. Molecular characterization of selected 35 endophytic bacteria by 16S rDNA–PCR–RFLP showed two main clusters at 48% and 43% similarity coefficients to which most of the endophytes belonged (Hung et al. 2007). In a recent study, Deng et al. (2011) isolated a total of 115 endophytic bacteria from root nodules of the wild legume *Sphaerophysa salsula* grown in two ecological regions of Loess Plateau in China. Of these, 50 strains were found as symbiotic bacteria (using nodulation test) belonging to eight putative species in the genera *Mesorhizobium*, *Rhizobium,* and *Sinorhizobium*, harboring similar *nifH* genes, as determined by RFLP and sequencing of 16S rRNA gene and enterobacterial repetitive intergenic consensus-PCR. *Mesorhizobium gobiense* was the main group while 65 nonsymbiotic bacterial strains related to 17 species belonged to the genera *Paracoccus*, *Sphingomonas*, *Inquilinus*, *Pseudomonas*, *Serratia*, *Mycobacterium*, *Nocardia*, *Streptomyces*, *Paenibacillus*, *Brevibacillus*, *Staphylococcus*, *Lysinibacillus,* and *Bacillus*. Interestingly, both symbiotic and nonsymbiotic bacterial strains coexisted in the nodules.

Endophytic bacteria, of hyperaccumulator origin, in contrast have shown a higher level of resistance to HM compared to those found in conventional soil and the rhizosphere environment. Endophytes are well adapted to higher concentrations of HM probably because they inhabit the interior tissues (Idris et al. 2004). In addition, endophytic populations occupying different organs of plants exhibit variable tolerance to HM. For example, methylobacteria are the most widespread in the hyperaccumulating plant endosphere and endorhizae. Methylobacteria, accounted for 20% of the endophytic community of hyperaccumulator plant Alpine Pennycress (*Thlaspi caerulescens*) grown in soil treated with a high content of zinc (Lodewyckx et al. 2002). Other authors have also demonstrated that endophytic methylobacteria (*Methylobacterium* sp. V3, *M. mesophilicum,* and *M. extorquens*) were found in *T. caerulescens* (Idris et al. 2006). Moreover, new species of methylobacteria (*M. goesingense*) was isolated from stems of *T. geosingense* (Idris et al. 2006), where *M. goesingense* forms resistant populations and often dominates in plants along with representatives of the genus *Sphingomonas*. These bacteria are recorded in the endosphere and endorhizae of Alpine Pennycress and a willow (*Salix caprea*) (Kuffner et al. 2010).

Despite the high concentration of HM in plant tissue, endophytic communities of any hyperaccumulator plant group have been reported to dwell in a wide range of bacteria. Apart from methylobacteria and sphingomonads, bacteria belonging to six other genera were also detected from interior part of stems of Alpine Pennycress, grown in the nickel-polluted soil. Of these, α-, β-, and γ-proteobacteria accounted for 67% of endophytic bacteriome (Idris et al. 2006). Later on, Barzanti

et al. (2007) reported mostly Gram-positive bacteria belonging to genera *Bacillus*, *Paenibacillus*, *Leifsonia*, *Curtobacterium*, *Microbacterium*, *Micrococcus,* and *Staphylococcus* in different parts of *Alyssum bertolonii* (a nickel hyperaccumulator, endemic to Central Italy serpentine soils). Only two groups of *Pseudomonas*-like bacteria were found as Gram-negative bacteria. Endophytes, such as *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes/Chlorobi*, were abundant in the endosphere of a willow and were resistant to Zn and Cd (Kuffner et al. 2010). The phylogenetic analysis of copper-resistant endophytic isolates from *Elsholtzia splendens* demonstrated that they belonged to three phylotypes: *Firmicutes*, *Actinobacteria,* and *Proteobacteria*, while *Bacillus* and *Acinetobacter* dominated the plant tissues (Sun et al. 2010). Interestingly, most of the endophytes studied so far have been shown to exhibit resistance to multiple HM (Lodewyckx et al. 2002). Such property of resistance to combination of metals at one time by endophytic bacteria indicates that prokaryotes in general have evolved various mechanisms to circumvent metal toxicity. Even though the resistance to certain combinations of HM is widespread in the natural environment, for certain mixture of metals like Ni with Co, the resistance is rare. The resistance to combination of Ni and Co is mediated by the *cnr* genes similar to those found in multiresistant bacteria *Cupriavidus metallidurans* (Liesegang et al. 1993). However, there are neither orthologs nor paralogs of known proteins for HM resistance in the indicated endophytes.

Heavy metal-resistant bacteria contain a small number of cation influx systems and perfect detoxification systems in cell-cytoplasm metal-binding proteins, and efflux pumps, which they have acquired through horizontal gene transfer (von Rozycki and Nies 2009). A selection of the HM-resistant endophytes by plants in response to the soil contamination takes place, and it results in a better plant accommodation in a specific niche. According to our results, the endophytic bacterial community of a French marigold (*Tagetes patula* L.), grown in a rocky substrate anorthosite, was different in structure and in ratio of cultivable forms of bacteria (Fig. 12.1a, b) compared to ones grown in the soil (Fig. 12.1c). There was a decrease in the bacterial species diversity, probably due to the toxic effects of HM released from rocks. Moreover, plants with the symptoms of intoxication (due to a high level of HM accumulation) and relatively healthy plants had significant variations in the endophytes, compared to marigold grown on an anorthosite. The poor growth, visual signs of metal toxicity on marigold plants and variation in endophytes populations correlated with higher level of HM accumulation (Fig. 12.2). However, the overall increase in the health of inoculated marigold plants was probably due to the metal detoxifying ability of the endophytic bacteria. In line with these findings, bioremediation ability of a multimetal-resistant endophytic bacterium *Bacillus* sp. L14 (EBL14) isolated from the cadmium hyperaccumulator *Solanum nigrum* L. was tested for heavy metals like Cu (II), Cd (II), and Pb (II) using 10 mg/l of each metal (Guo et al. 2010). Within 24 h incubation, the metal uptake by EBL14 was 76%, 80%, and 21% of Cd (II), Pb (II), and Cu (II), respectively, at the initial concentration of 10 mg/l, but there was no uptake chromium. Further, it was observed that the remediation efficiencies of strain EBL14 may profoundly be
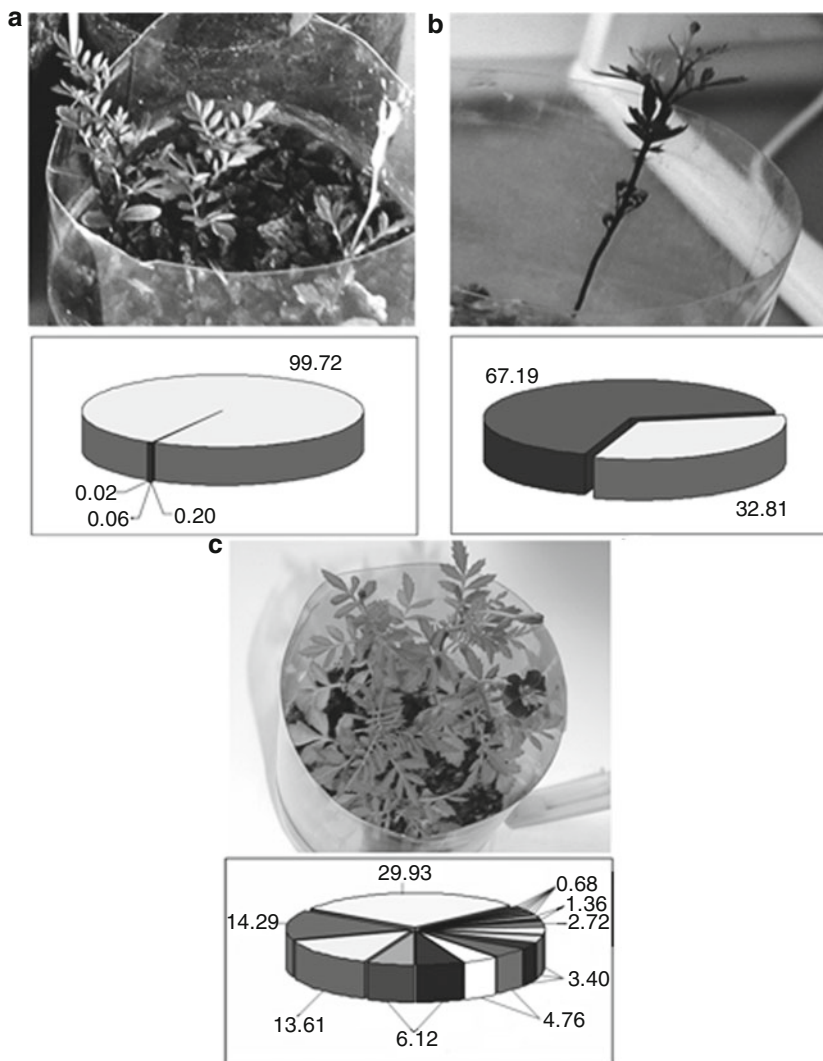
Fig. 12.1 The diversity of cultivable endophytic bacteria of *Tagetes patula* L. grown in the anorthosite rock (**a**, **b**) and soil (**c**) within a period of 70 days (% of total amount). Variants: relatively healthy plant (**a**) and intoxicated plant (**b**)

increased by inhibiting the activities of ATPase. In a follow-up study, Luo et al. (2011a, b) demonstrated that the cadmium removal by *Bacillus* sp. L14 (EBL14) increased from 74% (in the absence of DCC or DNP) to 94% and 81%, respectively. Further analysis of total and intracellular Cd concentrations after 24 h growth indicated that the enhanced Cd removal was due to the inhibitory effect of DCC or
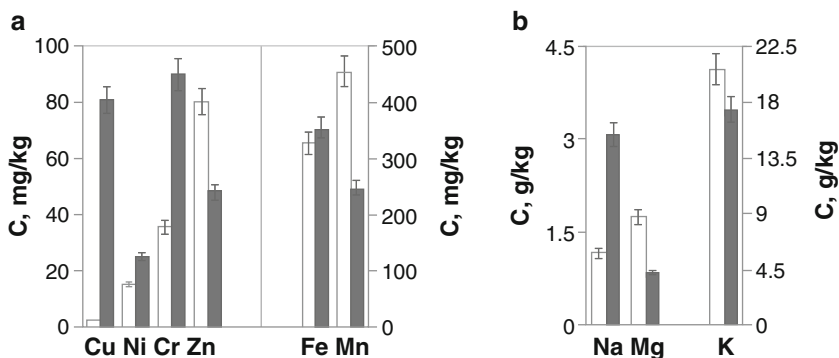
Fig. 12.2 The content of chemical elements accumulated by *Tagetes patula* L. grown in the anorthosite rock within the period of 70 days (C, mg/kg or g/kg dry mass). Variants: *open square*—relatively healthy plant; *filled square*—intoxicated plant. Data are presented as average mean ± standard deviation

DNP on the cations export resistance system of EBL14. Thus, the exceptional qualities and metal removal abilities of endophytes suggested that they could be used to develop inoculants for use in multimetal-contaminated soils.

12.3 The Alleviation of Metal Toxicity in Plants

12.3.1 Oxidative Stress Protection

At least three different molecular mechanisms of HM toxicity have been distinguished: (a) production of reactive oxygen species (ROS) like superoxide, hydroperoxyl radical, hydrogen peroxide, and hydroxyl radical species by autoxidation and Fenton reaction, (b) blocking of the essential functional groups in biomolecules, and (c) displacement of the essential metal ions from biomolecules (Schützendübel and Polle 2002). An exposure of plants to HM results in the oxidative stress as indicated by lipid peroxidation, H_2O_2 accumulation, and an oxidative burst (for details see Chap. 3). Heavy metals also cause a transient depletion of glutathione and an inhibition of antioxidative enzymes. The activation of the plant ROS-detoxification system by endophytes is considered as a promising way to protect plants from the toxic effects of HM. It is reported that the endophytic bacteria with their own ROS-eliminating system could complement the deficient antioxidative systems of the plant (Zaets et al. 2010; Ardanov et al. 2011). Both endophytic bacteria and fungi produce antioxidant compounds such as phenolic acids, flavonoids, tannins, hydroxyanthraquinones, and phenolic terpenoids (Jennings et al. 1998; Huang et al. 2007; Liu et al. 2009). On the other hand, endophytes either produce ROS themselves or stimulate peroxidative processes. According to White and Torres (2010), host plants could be protected by

endophytes through the enhanced tolerance to oxidative stress. They suggested that the enhanced antioxidant production by host plants may be the result of the production of ROS by endophytes. The endophytic inhabitants may permanently trigger the plant–host antioxidant system by ROS elicitors, keeping the system in the readiness to fight consequences of the environmental stressors' effects. Endophytes may activate the multiple plant antioxidant enzymes involved in combating oxidative stress caused by ROS during a plant defense (Ardanov et al. 2011). On the other hand, in order to survive under adverse conditions, endophytic inhabitants possess a variety of their own nonspecific tactics to fight against the production of ROS by plants, as well as nitric oxide and phytoalexins. The endophytic *Klebsiella pneumonia* 342, for example, has been shown to possess three superoxide dismutases, four putative catalases, six putative peroxidases, a hydroperoxide reductase, and 12 putative glutathione-*S*-transferase (GST) or GST domain/family proteins that can defend the cell against ROS released by plants (Fouts et al. 2008).

In our study, the plant guaiacol peroxidase (GPX) was activated at early developmental stages of soybean (*G. max*) and marigold when these plants were grown either in the metal-contaminated soil or on a rocky substrate. The GPX activity in both plants was, however, declined thereafter to a level of the control (Zaets et al. 2010). A similar increase in unspecific GPX was found in pine (*Pinus sylvestris*) roots (Schützendübel et al. 2001), barley (*Hordeum vulgare*) (Huang et al. 2006), and coontail (*Ceratophyllum demersum* L.) (Mishra et al. 2008) when grown in the presence of Cd. Inhibition of the GPX activity after a period of activation probably resulted in an increase of H_2O_2, and an oxidative burst activated the next stage of the antioxidant defense where the GST and insoluble phenolics were included. In the marigold leaves, the GST activity decreased during the budding and flowering, whereas in the soybean, it increased consistently which could probably be due to the varied defense mechanisms of plants. Changes in the enzyme activity and the phenolics content were more pronounced in the roots of plants since this organ is the first target of HM. Furthermore, the inhibition of the GPX activity of soybean roots was greater at lower Cd content than at a higher rate, following increase in the GST activity. Obviously, at lower metal concentration, the mechanisms that limit Cd uptake by upper part of plants work inefficiently. However, numerous studies have shown that the increase or decline in antioxidant enzyme activities depends on various factors like plant species, plant organs, and metal concentration (Pál et al. 2006; Rodríguez-Serrano et al. 2006; Schützendübel et al. 2001). It was assumed that under moderate stress conditions a plant responded by increasing the antioxidant enzymes' activities, but under extreme toxicity a general failure of the metabolism caused its attenuation. The activation of superoxide dismutase and inhibition of the GPX, catalase, and ascorbate peroxidase activities (as a result of blocking their SH-groups) results in H_2O_2 accumulation and causes an oxidative burst (Schützendübel et al. 2001).

Pretreatment of seed with bacteria protects plants from intoxication, probably, due to the cooperation of bacterial and plant antioxidant systems (Liu et al. 2009; Zaets et al. 2010). In the inoculated plants, changes in the peroxidase activity have

been found faster than in noninoculated plants. Maximal inhibition of the GPX was observed at an earlier stage of development as compared to untreated control plants, indicating stimulation of plant immunity by bacteria. The decrease in activity of the GST in shoots was likely to be associated with the increased enzyme activity in roots due to low metal transport in the above-ground parts of the plant. On the other hand, bacterial inoculation stimulates both production and accumulation of phenolic compounds by plant–hosts, known as antioxidants and chelators of HM, in the plant roots. An increase in the phenolics content was more expressed in marigold grown in the rocky substrate anorthosite at early development stage, whereas in soybean, grown in the cadmium contaminated soil, it was seen at both budding and flowering stages, as compared to the untreated plants where production of phenolics did not change (Zaetz and Kozyrovska 2008).

12.3.2 Indirect Reduction of Heavy-Metal Toxicity

Endophytic bacteria could contribute to the plant HM resistance indirectly by increasing the overall fertility of the contaminated soil and by providing plants with the additional nutrients, such as N, P, and Fe. As a result of this activity, the growth and health of plants are improved. One of the mechanisms of the plant growth promotion by endophytes is the nitrogen fixation. Endophytic diazotrophs, belonging to genera *Azoarcus*, *Herbaspirillum*, *Azospirillum*, *Gluconacetobacter*, *Klebsiella*, *Burkholderia*, etc., have been isolated from many important crop plants (Belimov et al. 2001; Gyaneshwar et al. 2001; Potrich et al. 2001; Muthukumarasamy et al. 2002). These bacteria are often found inside roots and/or the dense plant tissue (stem nodes and xylem vessels); the bacteria are likely to be growing within a low pO_2 environment which is necessary for the expression and operation of nitrogenase (Baldani et al. 1997). The endophytic *Azospirillum* spp. isolated from the roots of plants grown on contaminated sites varied substantially in their in vitro tolerance to Zn and Cd (Moreira et al. 2008). There is no information available about the correlation between the Nif$^+$ phenotype of endophytes and HM resistance; however, the N obtained from N_2 fixation improves plant growth in polluted sites and, therefore, supports the plant defense system. Certain endophytic bacteria also solubilize insoluble P by producing acids and concomitantly increase the availability of soluble P and other nutrients to plants in nutrient deficient soils (Verma et al. 2001; Zaetz et al. 2006). Kuklinsky-Sobral et al. (2004), for instance, showed that 52% of the endophytic bacteria isolated from a soybean rhizosphere could solubilize P. In metal-contaminated soils, plants are typically unable to accumulate sufficient Fe despite the phytosiderophores' secretion or production of organic acids, lowering the pH of the soil and increasing Fe availability. This is because of the fact that plant siderophores generally have a lower (by 10–30 orders of magnitude) affinity for Fe than do the bacterial siderophores. Therefore, bacteria capable of producing siderophores are likely to protect plants from Fe-deficiency. The bacterial siderophore production may be stimulated by the presence of HM and, probably, help the plant to reduce heavy-metal toxicity by increasing the supply of

iron to the plant (Burd et al. 2000). Barzanti et al. (2007) reported that 83% of endophytic isolates (mainly Gram-positive bacteria) of *A. bertolonii* produced siderophores and stimulated the plant growth under the Ni-induced stress. In contrast, only 2% of willow endophytes resistant to Zn and Cd synthesized siderophores (Kuffner et al. 2010). We, therefore, deduce that by providing a plant even with low level of available nutrients could be one of the bacterial effects involved in the overall defense mechanism against HM.

12.3.3 Phytohormone-Mediated Defense Effects

Endophytic bacteria have been shown to have a plant growth-promoting activity that can be due to the production of phytohormones and enzymes such as ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, auxins, indole-3-acetic acid (IAA), acetoin, 2,3-butanediol, cytokinins, etc. For example, auxins and cytokinins are reported to be produced by endophytic strains of *Pseudomonas*, *Enterobacter*, *Klebsiella,* and *Azospirillum* (Kozyrovskaya et al. 1990; Leifert et al. 1994; Bashan and Holguin 1997) and in some cases approximately half of endophytic isolates resistant to HM were able to produce IAA (Sheng et al. 2008). The authors concluded that ability of bacteria to tolerate HM stimulated the growth of plant roots in metal enriched soils. Ethylene-degrading enzyme ACC deaminase also plays a critical role in acquiring resistance to HM. Heavy metals induce the production of ethylene in plants, which at higher concentration inhibits plant development (Weckx et al. 1993). The bacteria that hydrolyze the precursor of ethylene protect plants from ethylene stress. Idris et al. (2004), for example, reported that 36% of endophytes of *T. goesingense* synthesized ACC deaminase. However, this percentage is not too big, and its contribution to the prevention of the ethylene emission may not be significant. In other study, Madhaiyan et al. (2007) showed that *M. oryzae* and *Burkholderia* sp. (isolated from the rice tissues) were able to protect tomato (*Lycopersicon esculentum*) seeds from Ni and Cd toxicity by lowering the stressful level of ethylene. Abscisic acid (ABA) and jasmonic acid have also been reported to be produced by endophytic *Bacillus pumilis* isolated from sunflower (Forchetti et al. 2007). Both ABA and jasmonates are the key hormones that provide defense to plants against abiotic stresses (Yasuda et al. 2008). Phytohormone production by bacteria may affect hormone-regulated processes in plants, including a plant defense system. Usually, priming of the plants leads to the induction of a systemic disease resistance by endophytes in host plants via SA- or jasmonate-ethylene-dependent signaling pathways (Iniguez et al. 2005; Conn et al. 2008; Ardanov et al. 2011). The structure of endophytic bacterial communities in plants may, however, be changed following activation of plant defense system in the stressed environment, and we may foresee that, probably, the environmental stressors like HM activate the endophytic microbial communities for combating a danger directed against both their survival and their interaction with host plants.

12.4 Endophytic Bacteria: Importance in Phytoremediation Technologies

The ability of plants to extract and degrade harmful substances or HM through a process generally called as phytoremediation has been considered as a substitute to chemical technologies in metal cleanup program. Endophytes in this context exert positive effects on plants through various mechanisms and are reported to enhance phytoremediation efficiency (Rajkumar et al. 2009). Endophytes have been shown to enhance in some cases the accumulation of HM, and so far their potential to improve phytoremediation is huge. Moreover, endophytic bacteria can also assist their host plants in overcoming the phytotoxic effects of HM by other mechanisms as well. Collectively, plant growth and development, improving metal bioavailability and translocation, enhancement of metal uptake, and reduction/removal of metal toxicity to plants are the hallmarks of endophytes when applied properly for polluted environment. In addition, endophytes when used in phytoremediation programs have advantage over both soil and the rhizosphere bacteria because of their ability to establish intimate relationships with corresponding host plants and their ability to facilitate growth and health of plants.

There have been attempts in recent years to modify endophytic strains genetically in order to produce phenotype of the HM-resistance; however, the results on HM-accumulation have not been conclusive. For example, the nickel-resistant endophytic bacteria *Burkholderia cepacia* and *Herbaspirillum seropedicae* engineered by transferring the resistance genes *ncc-nre* (Ni–Co–Cd) from *C. metallidurans* 31A showed a variable uptake potency, when used as inoculant for yellow lupine (*Lupinus luteus*) (Lodewyckx et al. 2001). Plants inoculated with modified *B. cepacia* L.S.2.4::*ncc-nre* did not accumulate Ni in the above-ground biomass, and *H. seropedicae* even reduced the level of Ni accumulation in a rye grass (*Lolium perenne*). In contrast, the recent report of Weyens et al. (2010) encouraged the genetic manipulations with bacteria to optimize the phytoremediation of co-contaminations by organic pollutants and toxic metals. In model experiments, a yellow lupine was inoculated with the endophyte *B. cepacia* VM1468, possessing trichloroethylene (TCE) degradation potency due to the pTOM-Bu61 plasmid, carrying the *ncc-nre* genes. Inoculation of lupine with *B. cepacia* VM1468 resulted in decreased Ni and TCE phytotoxicity and a five times higher Ni uptake. Endophytic actinobacteria, associated with the HM-accumulating willow, have the potential to increase Zn and/or Cd uptake and may be applicable in phytoremediation (Kuffner et al. 2010). Added to the Cd-amended soils, *Serratia nematodiphila*, *Enterobacter aerogenes*, *Enterobacter* sp., and *Acinetobacter* sp., isolated from Cd-hyperaccumulator *Solanum nigrum* L., significantly increased Cd extraction from the soils and influenced the accumulation of Cd in the root, stem, and leaf tissue of *S. nigrum* L. Under these conditions, strains could colonize the plant interior tissues. It was concluded that experimental bacteria could be exploited to improve the efficiency of phytoextraction (Chen et al. 2010).

Conclusion

Understanding the basic mechanisms by which endophytes support the metal accumulation process and their interaction with plants are likely to offer great practical benefits in the remediation of polluted sites and consequently higher biomass yields of crops. Currently, this is, however, not clear, how HM-resistant endophytes affect the resistance of plants and what the mechanism of such influence is, if it exists. Probably, the resistance of endophytes to HM is not related to the accumulation of metal cations by hyperaccumulator plant, since they have sometimes a reduced ability to uptake the latter. Despite ample progress, further studies are needed to substantiate endophytic bacteria-assisted phytoremediation. The availability of the genome sequences of both host plants and associated endophytes combined with metabolome, transcriptome, and other "oms" analyses is likely to provide a better understanding of the synergistic relationships between them, as well as the construction of metabolic pathways for improving the relatively inefficient remediation technologies.

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Importance of Arbuscular Mycorrhizal Fungi in Legume Production Under Heavy Metal-Contaminated Soils

13

Diriba Muleta and Delelegn Woyessa

Abstract

Degradation and loss of arable lands have attracted attention of the scientists worldwide to protect lands from further declining. The multifaceted functions of legumes on the other hand in the improvement of natural and managed terrestrial ecosystems have necessitated their sustainable production. Leguminous crops are the important protein source in human dietary systems particularly in developing countries. The legume productions have, however, been seriously hampered due to heavy metal contamination of soils. Maintenance of soil quality adopting various remediation strategies including biological approaches is therefore important. Mycorrhizoremediation among bioremediation has currently been the focus of research. Arbuscular mycorrhizal fungi (AMF) establish mutual symbioses with the majority of higher plants and after colonization, contribute to the plant growth in metal-contaminated sites by increasing plant access to P, by improving soil quality, and by restricting the movement of metals within plant tissues. Here, we have focused to understand on how the heavy metals affect legumes and the incidence of AMF in metal-polluted sites. In addition, the role of AMF in restoring heavy metal-contaminated sites is described. Understanding the role of mycorrhizae in metal detoxification is likely to improve the agronomic strategies in order to take full advantage of mycorrhizal association for legume production in disturbed cropping locations.

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13.1   Introduction

Legumes are important plant group that contributes substantially to nitrogen (N) pool and the productivity of many terrestrial ecosystems (Cleveland et al. 1999). Over the years, legumes have been grown for the food, feed, forage, fiber, industrial and medicinal compounds, and other end uses. Moreover, legumes are also highly suitable for agroforestry system. Nevertheless, productions of legumes have seriously been restricted due to environmental pollutions. In addition, humankind faces multiple anthropogenic global environmental challenges, which are now large enough to exceed the bounds of natural variability (Clark and Tilman 2008). Currently, increasing heavy metal (HM) contamination due to various human and natural activities has exceedingly compromised the quality and functions of ecosystems. Heavy metals occur mainly in terrestrial or aquatic ecosystems although they can also be emitted into the atmosphere. Agroecosystems receive heavy metals from (1) the increased use of commercial fertilizers and biocides; (2) the application of metal-containing wastes such as sewage sludge, pig manure, coal and wood ashes, and soils; (3) atmospheric deposition; and (4) emissions from municipal waste incinerators, car exhausts, and smelting industries (Liu et al. 1997). These toxicants are included in the main category of environmental pollutants as they persist in the environment. Excessive HM accumulation is, however, hazardous to humans, animals, and plants (Panda 2008).

Evidently, heavy metals are extremely resistant to chemical degradation and need to be physically removed or immobilized (Kroopnick 1994). Conventionally, remediation of heavy metal-contaminated soils involves either on-site management or excavation and subsequent disposal to a landfill site (Parker 1994). However, this method of disposal merely shifts the contamination problem to a different place along with the hazards associated with transportation of contaminated soil and migration of toxicants from landfill into an adjacent environment. Soil washing for removing contaminated soil is a substitute to excavation and disposal to landfill (Elliott et al. 1989). This method is also costly and generates metal-rich residues which will require further treatment or burial procedures. Apart from these, physicochemical technologies make the land unsuitable for cultivation since along with the contaminants, they also remove biological activities from polluted sites. Consequently, this demands the development of sustainable on-site techniques for biological remediation of heavy metal-contaminated sites.

Bioremediation in this context is a suitable option that may involve the use of living organisms to restore or clean up contaminated soils. This technique is attractive due in part to its convenience and low operation cost (Leyval et al. 2002). The use of plants in particular for bioremediation purpose is based on their ability to uptake and accumulate HMs. The success of bioremediation, however, depends on three important factors: (1) availability of microbes, (2) accessibility of contaminants, and (3) a conducive environment (Brar et al. 2006). Plants among the biological resources are more dependent on microbial activity since microorganisms enhance the metabolic activities of plants to combat stresses in polluted areas (Killham and Firestone 1983). Therefore, microorganisms and their interactions in soil play a critical role in nutrient

transformations and cycling, and in sustaining soil fertility (Nayyar 2009). In addition, the rhizosphere microbial populations are also known to affect heavy metal mobility and availability to the plants through release of chelating agents, acidification, phosphate solubilization, and redox changes. Consequently, microbes enhance phytoremediation processes.

Among the microorganisms that affect rhizosphere processes, symbiotic fungi forming mycorrhizae induce a series of changes in plant physiology, nutrient availability, and microbial composition that may determine the outcome of a phytoremediation performance. Recognition of the intertwined relationship of plants and symbiotic nonpathogenic fungi collectively called as mycorrhiza (Harley and Smith 1983; Sieverding 1991) therefore opens up a new horizon for sustainable cleaning up of the seriously polluted environments. Concomitantly, mycorrhiza becomes a significant component in low agriculture production systems (Barea and Jeffries 1995). Arbuscular mycorrhizal fungi are abundant soil microorganisms and are considered as important functional components of the soil–plant system occurring in many ecological niches (Brundrett et al. 1996; Makoi and Ndakidemi 2009). Furthermore, AM fungi when used either alone or in combination with other rhizosphere microbes can enhance plant growth including legumes both in conventional (Zaidi et al. 2003; Zaidi and Khan 2006) and in HM-contaminated sites, by increasing plant access to relatively immobile minerals such as P (Yao et al. 2003; Marschner and Dell 1994), by improving soil texture by binding soil particles into stable aggregates that resist wind and water erosion (Degens et al. 1996 ; Rillig and Steinberg 2002; Steinberg and Rillig 2003; Farahani et al. 2008), and by binding heavy metals into roots that restricts their translocation into shoot tissues (Kaldorf et al. 1999). Arbuscular mycorrhizal fungi can accelerate the revegetation of severely degraded lands such as coal mines or waste sites containing high levels of heavy metals (Marx and Altman 1979).

It is considered that AMF might have evolved long before legumes; it is now assumed that all legumes have the potential to form symbiosis with AMF. Truly, it has been demonstrated that legumes are generally more mycotrophic than other plants (Plenchette et al. 2005) since AMF form symbiotic association with an array of members of family *Fabaceae* (or *Leguminosae*) (Pagano et al. 2007; Valsalakumar et al. 2007; Molla and Solaiman 2009), suggesting efficient way of remediation role using such phytobeneficial soil microbes. Mycorrhizal dependency of legumes plants has recently been reviewed (Muleta 2010). In legume plants, the importance of AMF symbiosis has been attributed to high P requirements on the nodulation and N_2 fixation process which requires enhanced P uptake (Barea and Azcon-Aguilar 1983). It was reported that P is a critical element in forming nodules (Toro et al. 1998). Improved P nutrition has been shown to increase in infertile and P fixing soils of the tropics following AM inoculation (Dodd 2000; Zaidi et al. 2003). Mycorrhizae have also been reported in plants growing on HM-contaminated sites, indicating that these fungi have evolved mechanism to tolerate heavy metals. Therefore, these fungi may play a role in the phytoremediation of the contaminated site (González-Guerrero et al. 2009). For example, Jamal et al. (2002) demonstrated that members of Glomeromycota had the ability to ameliorate the toxicity of HM-polluted soils

where various leguminous plants were grown. Interestingly, there are reports which suggest that dual inoculation of legumes with AMF and bacteria resulted in remarkable tolerance to heavy metal toxicity (Vivas et al. 2003a, b; Muleta 2010). For heavy metal resistance, AMF involve a range of mechanisms such as sequestration and accumulation of heavy metals in AM fungal biomass and in the roots of host plants (Joner et al. 2000a, b). Other mechanisms of heavy metal tolerance by mycorrhizal legumes have also been reported (Malcova et al. 2003; Cardoso and Kuyper 2006; Garg and Aggarwal 2011).

13.2 Negative Influence of Heavy Metal Toxicity to Legumes

Legumes are highly important functional group of plants which determines the N economy of varied ecosystems (Makoi et al. 2009). Since the N is one of the major nutrients for plant growth, the deficiency of N or inhibition of N uptake induced by heavy metals will have a strong negative impact on plant's performance. Heavy metals are a group of metal elements with specific weight greater than 5 g cm^{-3} (Weast 1984). Unlike certain mineral elements like, Fe, Cu, Mn, and Zn, which are considered essential for normal plant growth as they are required in numerous enzyme-catalyzed or redox reactions, in electron transfer, and have structural function in nucleic acid metabolism, HMs such as Cd, Pb, Hg, and As are not essential for normal plant growth (Mertz 1981). Further, Hall (2002) and Järup (2003) have demonstrated that at elevated levels, HMs are lethal to most organisms. The nonessential heavy metals enter the root system via passive diffusion or by low-affinity metal transporters with broad specificity (Hall and Williams 2003) and depressingly affect plant growth (Shetty et al. 1995).

The presence of higher concentration of heavy metals in agricultural soils is a major concern since they may have long-term effects on soil functioning (Tyler et al. 1989). In contrast, considerable amounts of HMs such as mercury may however, be added to agricultural land with fertilizers, lime, and manures. The most important sources of contaminating the agricultural soils have been the use of organic mercurials as a seed-coat dressing to prevent fungal diseases in seeds. At high concentrations, HMs interfere with essential enzymatic activities by modifying protein structure or by replacing a vital element, resulting in deficiency symptoms. The plasma membrane is particularly vulnerable to HM toxicity since membrane permeability and thus functionality can be affected by alterations of important membrane intrinsic proteins such as H$^+$-ATPases (Hall 2002). For instance, toxic effects of mercury in plants include abscission of older leaves, growth reduction, decreased vigor, inhibition of root and leaf development, and decreased chlorophyll content and nitrate reductase activity (Vyas and Puranik 1993). Other adverse effects caused by excessive mercury include membrane structure integrity disruption (Ma 1998), mineral nutrient uptake reduction (Patra and Sharma 2000), and photosynthesis and transpiration reduction (Krupa and Baszynski 1995).

Leguminous plants on the other hand have advantages like they are capable of transforming atmospheric N into usable form of N (e.g., ammonia) by forming

nodules on legume root system (Ma et al. 2006). However, root nodules formation may be inhibited by heavy metals in the soil, and thus the N_2-fixing ability of leguminous plants may be hindered. For example, higher concentrations (>1–$2\,mg\,l^{-1}$) of mercury decreased the growth of alfalfa plants (Zhou et al. 2007). Mercury also inhibited water uptake through aquaporins in plasma membrane in wheat (Zhang and Tyerman 1999). Inhibition of enzymes of different metabolic pathways has also been reported by mercury toxicity (Morch et al. 2002). Among other metals, Cd has been reported to severely inhibit the biomass production (Leita et al. 1993), photosynthetic activity (Kumar and Kumar 1999), and nitrogen fixation and nodulation (Hernandez et al. 1995), leading consequently to reduced uptake of nutrient elements (Obata and Umebayashi 1997) in pea (*Pisum sativum*). In a follow-up study, Rivera-Becerril et al. (2002) reported a decrease in shoot, root, and pod biomass of pea grown in soils treated with Cd. The prolonged period of Cd stress has been found to cause the decrease in water availability which in turn changes the concentrations of many metabolites, and disrupt amino acid and carbohydrate metabolism (Liao et al. 2005). On the other hand, in many species of plants, the level of metal accumulation can exceed even several hundred times the maximum level accepted for human beings, without a visible negative symptom on their growth or yield (Wierzbicka and Obidzinska 1998). Therefore, it seems that plants can endure a level of environmental pollution that might be even several times higher than the level observed nowadays (Gupta et al. 2007; Murakami and Ae 2009). However, several adverse ecotoxicological effects of heavy metals on plants and microorganisms in general and legumes in particular at varying degrees have been well reported. For instance, there are reports of adverse effects of heavy metals on nodulation and N_2 fixation of clover (*Trifolium pratense*) (McGrath et al. 1988) and chickpea (*Cicer arietinum*) (Yadav and Shukla 1983). Al-Garni (2006) in a report has also confirmed that the nodule formation and percentage of mycorrhizal infection in cowpea were greatly reduced with increasing Zn and Cd concentrations (Fig. 13.1), but Cd was found more toxic and inhibitory than Zn to inoculated plants. Giller et al. (1989), in a similar study, also suggested that either of the two or both mechanisms acting simultaneously can limit N_2 fixation: (1) one or more of the metals could prevent the formation of N_2-fixing nodules by effective *Rhizobium* strains inhabiting soil, or (2) the metal contamination could cause the elimination of the effective *Rhizobium* strains from the polluted soil.

Furthermore, the HMs are known to alter important plant membrane's intrinsic proteins such as H^+-ATPases (Hall 2002) and produce reactive oxygen species (ROS) which potentially damage plant tissues (Schutzendubel and Polle 2002), leading to chlorosis, growth retardation, browning of roots, and other harmful effects on the photosystems. A growing body of evidence (Gildon and Tinker 1981) reveals that heavy metals can delay, reduce, and even completely eliminate AM colonization and AMF spore germination in the field, and a negative correlation between Zn concentrations and AM colonization has been reported in soil treated with urban industrial sludge (Boyle and Paul 1988), clearly indicating the serious negative impacts of HMs on plant function and distribution.
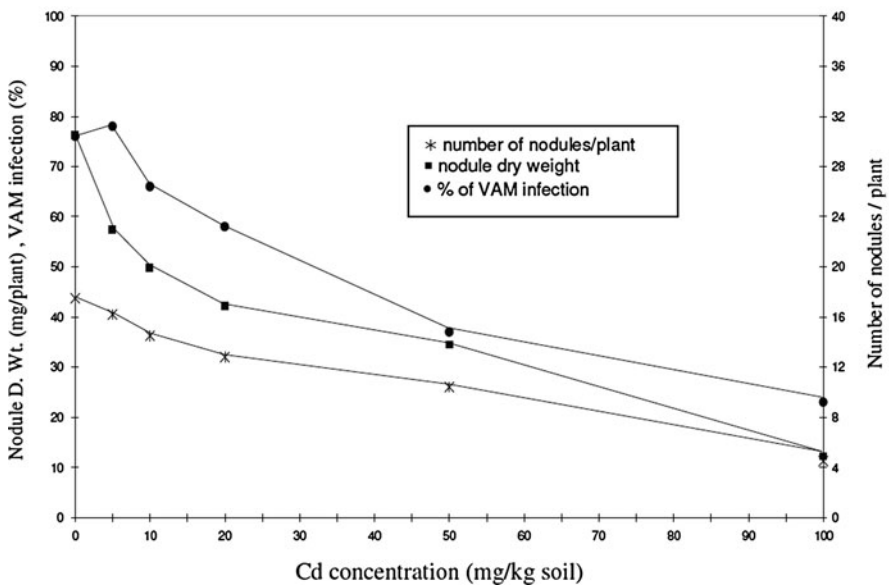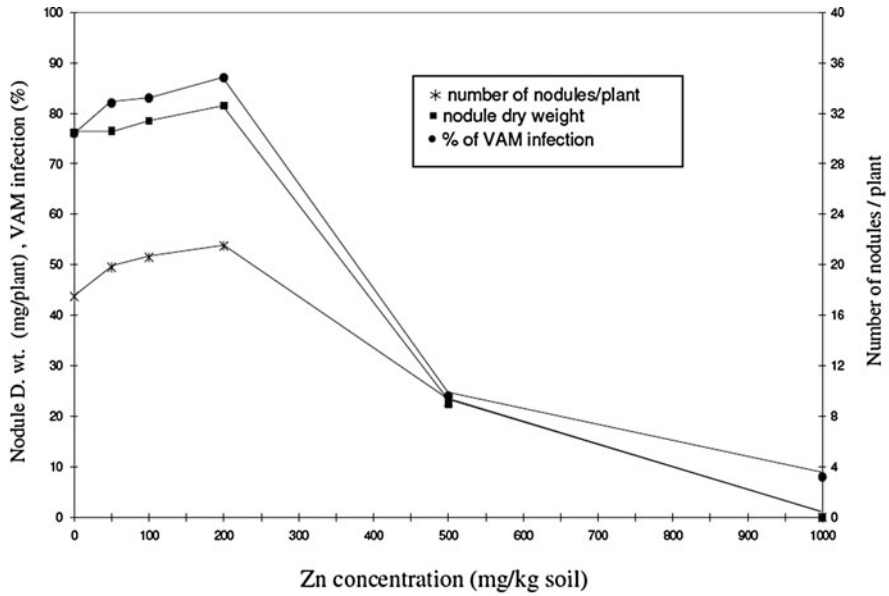
Fig. 13.1 Effect of different concentrations of zinc and cadmium on root nodulation of plants (adapted from Al-Garni 2006)

13.3 Incidence of Arbuscular Mycorrhizal Fungi in Metal-Polluted Sites

Arbuscular mycorrhizal fungi inhabit almost all habitats and climates (Barea et al. 1997), including disturbed soils such as those derived from mine activities (Brundrett et al. 1996). For instance, Gaur and Adholeya (2004) reported that AM fungal species can be isolated from areas naturally enriched by heavy metals or old mine/industry waste sites. Accordingly, several heavy metal-tolerant AM fungi have been isolated from polluted soils (Turnau and Mesjasz-Przybylowicz 2003; Leung et al. 2007). Such metal-tolerant AM fungi have a positive implication since they can be used for reclamation of such degraded soils. Various mycorrhizal fungi such as *Glomus* and *Gigaspora* (Raman et al. 1993; Raman and Sambandan 1998), *G. fasciculatum* (Dueck et al. 1986), *G. aggregatum*, and *Entrophospora* spp. (Pawlowska et al. 1996) were found associated with most of the plants growing in heavy metal-polluted habitats. Further, Gildon and Tinker (1981) isolated a mycorrhizal strain which tolerated 100 mg Zn kg^{-1} soil. Considerable amount of AM fungal colonization was also reported in an extremely polluted metal mining area with HCl-extractable Cd soil concentration of more than 300 mg kg^{-1} (Gildon and Tinker 1983). Similarly, Weissenhorn et al. (1993) isolated mycorrhizal fungi from two heavy metal-polluted soils, which were found to be more resistant to Cd than a reference strain. In India, Sambandan et al. (1992) reported 15 AM fungal species from heavy metal-contaminated soils. del Val et al. (1999a) have demonstrated the diversity of AM fungi in sewage-amended sludge containing heavy metals in a long-term experiment. The results indicated that six AM fungal species belonging to the genus *Glomus* found in rhizosphere samples from the different experimental trap plants and soil treatments were *G. claroideum*, *G. mosseae*, and four additional, unidentified species numbered III to VI. *G. claroideum* and *Glomus* sp. V were the most common fungi in the rhizospheres of all host plants growing in soils treated with 300 m^3 ha^{-1} per year of contaminated sludge. In this perspective, AM fungi constitute an important functional component of the soil–plant system that is critical for sustainable productivity in degraded soils.

13.4 Variation Among AMF to HM(s) Tolerance

Soil degradation and HM contaminations produce changes in the diversity and abundance of AM fungal populations and consequently interfere with possible beneficial effects of the mycorrhizal association. The elimination of AM fungal populations from such soils can, therefore, have a negative impact on plant establishment and survival (Pfleger et al. 1994), which otherwise is important (Haselwandter and Bowen 1996). On the other hand, there are reports of the presence of AM fungi in metal-contaminated soils, which suggests the adaptive habit of the indigenous AM populations. Different AMF ecotypes have, however, been shown to differ in their susceptibility and tolerance to heavy metals

(Leyval and Weissenhorn 1996). Consequently, marked variation in the effectiveness of AM root colonization in terms of nutrient acquisition is reported (Ahiabor and Hirata 1995). Weissenhorn et al. (1995) suggested a high tolerance of indigenous AM fungal population to elevated metal concentrations in soil and inside the roots. AM fungal colonization up to 40% was reported in spite of high Cd (1,220 mg kg^{-1}) and Pb (895 mg kg^{-1}) concentrations. They further reported abundance of AM fungi (100 spores per 50 g soil) in two agricultural soils close to a Pb–Zn smelter. Of the 15 AM species isolated from heavy metal-contaminated soils in India, *Glomus geosporum* was found in all the sites studied (Sambandan et al. 1992). However, in addition, the total number of AM fungal spores decreased with long-term sludge application and with increasing amounts of heavy metals. The AM fungal spores never disappeared completely in soils amended with the highest rates of sludge, suggesting a certain adaptation of these indigenous AM fungi to such environmental stress (del Val et al. 1999a). Six AM fungal ecotypes were found in the experimental soils, showing consistent differences with regard to their tolerance to the presence of heavy metals. Other studies (Pawlowska and Charvat 2004) have also examined *in vitro* effects of Cd, Pb, and Zn on critical life stages in metal-sensitive ecotypes of AM fungi, including spore germination, presymbiotic hyphal extension, presymbiotic sporulation, symbiotic extraradical mycelium expansion, and symbiotic sporulation. Despite long-term culturing under the same low-metal conditions, two species, *Glomus etunicatum* and *G. intraradices*, had different levels of sensitivity to metal stress: *G. etunicatum* was more sensitive to all three metals than was *G. intraradices*. A unique response of increased presymbiotic hyphal extension occurred in *G. intraradices* exposed to Cd and Pb. Presymbiotic hyphae of *G. intraradices* formed presymbiotic spores, whose initiation was more affected by heavy metals than was presymbiotic hyphal extension. In *G. intraradices* grown in compartmentalized habitats with only a portion of the extraradical mycelium exposed to metal stress, inhibitory effects of elevated metal concentrations on symbiotic mycelial expansion and symbiotic sporulation were limited to the metal-enriched compartment. Symbiotic sporulation was more sensitive to metal exposure than symbiotic mycelium expansion.

Considerable variations have also been observed between indigenous and reference strains of AMF of dissimilar origin (i.e., contaminated or uncontaminated soils/commercial strains) with regard to their tolerance to heavy metal toxicity. Experimental results reveal that indigenous AM fungi showed tolerance to heavy metals as they germinated in soils containing 6,060 mg kg^{-1} Pb, 24,410 mg kg^{-1} Zn, and 1,630 mg kg^{-1} Cu (Gaur and Adholeya 2004). Likewise, AMF isolates, particularly the ecotypes living in metal-enriched soils, metalliferous sites, and mine spoils heavily polluted with metals, can, depending on intrinsic and extrinsic factors, tolerate and accumulate HMs (Weissenhorn et al. 1993; Joner and Leyval 1997; Smith and Read 1997). However, neither selection based on *in vitro* growth trials nor selection of presumably adapted fungi will guarantee success. For example, *in vitro* tolerance of an ectomycorrhizal fungus to Zn did not always predict its tolerance as a symbiont (Colpaert and Vanassche 1992).

13.5 The Role of AMF in Imparting Metal Tolerance to Legume Plants

Resistance refers to the ability of microorganisms to withstand the effects of a pollutant usually effective against them, while tolerance refers to the ability of microorganisms to adapt to the persistent presence of the pollutant (Giasson et al. 2008). The use of soil fungi to remediate or clean up contaminated soils can be viewed as a promising method to remove and/or stabilize soils contaminated with heavy metals (Hall 2002; Hildebrandt et al. 2007; Makoi and Ndakidemi 2009). Consequently, symbiosis with mycorrhizal fungi has been proposed as one of the most promising strategies for providing immunity to plants against metal toxicity (Upadhyaya et al. 2010). Metal tolerance ability of AM fungi and ectomycorrhizal (ECM) fungi has been assessed using several characteristics including AM spore numbers, root colonization, and the abundance of ECM fruiting bodies (Weissenhorn et al. 1993; del Val et al. 1999b). For example, mycorrhiza-colonized plants bioremediated the nickel-contaminated soil by 30% more than noncolonized plants (Turnau and Mesjasz-Przybylowicz 2003). The As and Ni bioremediation from the soil through colonized plants could have antagonistically increased soil P which could be available for plant growth and development (Liu et al. 2005; Leung et al. 2007). In cropping systems involving legumes such as pea (Makoi and Ndakidemi 2009) and clover (Medina et al. 2005), AM can provide mycorrhizal buffer to stress imposed by Cd or Cd tolerance by changing its polyamine metabolism, thus stabilizing Cd in the root system of colonized plants (Parádi et al. 2003).

A pot experiment was conducted to evaluate the role of AMF in reducing the toxicity of heavy metals (Abdel-Aziz et al. 1997). A newly reclaimed soil from Ismailia Governorate was supplemented with sewage sludge from two sources at five different rates: 0%, 0.5%, 1%, 2%, and 4%. One half of each treatment was inoculated with AMF. Application of sewage sludge up to 2% increased faba bean growth, nodule numbers and weight, and P and N contents. Inoculation with AM also induced significant increase in the measured parameters as compared with the uninoculated treatments. Concentration of Zn, Mn, Cu, Ni, Cd, Pb, and Co in plant was increased following sewage sludge application. However, in the sludge-treated soil which had high metal concentrations, AM inoculation reduced the concentration of metals, suggesting that AM could play an important role in reducing the hazardous effect of heavy metals, when present at higher levels.

The role of AM inoculation in understanding the effects of metals and metal tolerance in red kidney was determined by growing this legume in soil artificially contaminated with high concentrations of Zn, Cu, Pb, and Cd (Rabie 2005). Mycorrhizal red kidney plants accumulated relatively high metal concentrations in shoots which indicated that internal detoxification metal tolerance mechanisms are also active. From a number of physiological indices measured in this study, mycorrhizal symbiosis significantly increased root and shoot dry weight, protein content, and the activity of antioxidant enzymes in red kidney plants compared to the control. The beneficial effects of the AM fungus observed in this study generated an enormous interest in developing arbuscular mycorrhizal fungi for

plant-based metal remediation strategies. Following this message, Lin et al. (2007) conducted a greenhouse experiment to investigate the effects of AMF *Glomus mosseae* on the growth and metal uptake of three leguminous plants (*Sesbania rostrata*, *S. cannabina*, *Medicago sativa*) grown in multimetal-contaminated soils. AMF colonization increased the growth of the legumes, indicating that AMF colonization increased the plant's resistance to heavy metals. AMF inoculation also significantly stimulated the formation of root nodules and increased the N and P uptake of all of the tested leguminous plants. Compared with the control, colonization by *G. mosseae* decreased the concentration of metals, such as Cu, in the shoots of the three legumes, indicating that the decreased heavy metal uptake and growth dilution were induced by AMF treatment, thereby reducing the heavy metal toxicity to the plants. The root/shoot ratios of Cu in the three legumes and Zn in *M. sativa* were significantly increased with AMF colonization, indicating that heavy metals were immobilized by the mycorrhiza and that the heavy metal translocations to the shoot were decreased. To validate this further, Andrade et al. (2010) studied the influence of jack bean [*Canavalia ensiformis* (L.) DC] inoculation with AM fungus *Glomus etunicatum*, in response to increasing Cu concentrations (0, 50, 150, and 450 mg dm^{-3}) in soil. At the highest Cu dose (450 mg dm^{-3}), mycorrhiza decreased Cu concentrations in plant organs and increased biomass accumulation. In addition, mycorrhizal colonization was not affected by Cu, suggesting certain tolerance of the inoculated AM fungus to this metal. A number of similar research findings have also shown that AMF inoculation has remarkably improved the tolerance of leguminous plants to heavy metal toxicity under different growing regions (Chen et al. 2007). Collectively, AM fungi are able to maintain an efficient symbiosis with leguminous plants in soil containing high heavy metal concentrations. Microbial adaptation to heavy metal (e.g., Zn) has been attributed to two factors (Almås et al. 2004). The first one is a gradual decrease in metal availability due to immobilization reactions occurring in the rhizosphere. The second factor is a gradual change in microbial community structure, based on changes in phospholipid fatty acid profiles (Frostegård et al. 1993) which results in more tolerant organisms. More heavy metal tolerance has been associated with microbial consortia. For instance, Wang et al. (2005) have verified that the AM fungal consortium can benefit phytoextraction of HMs and therefore play a fundamental role in phytoremediation of HM-contaminated soils.

13.6 Composite Inoculation Effects of AM Fungi on Legume Plants Grown in Metal-Contaminated Soils

The results on the composite inoculation effect of mycorrhizal fungi and bacteria on legume plants under different growth conditions are well documented (Muleta 2010). Moreover, many studies show that composite inoculants prepared from different microbial groups may stimulate plant growth (Barea et al. 2002; Zaidi et al. 2003). It is assumed that the beneficial effect of AM fungi coinoculated with selected bacterial strains is due to the synergistic interactions capable of increasing shoot

and/or root growth, nutrient uptake, and plant tolerance to stresses (Vivas et al. 2003b). For example, the beneficial effect of an indigenous Cd-tolerant *G. mosseae* coinoculated with a Cd-adapted strain of *Brevibacillus* sp. in terms of improving plant tolerance to Cd contamination has been reported (Vivas et al. 2003a, b). Arbuscular mycorrhizal fungi when used alone enhanced legume growth and numbers of root nodules and led to higher N accumulation in the plants compared with the control (Lin et al. 2007). The increased nodulation and consequently N_2 fixation in mycorrhizal legumes was suggested due to better nitrogenase activity (Chen et al. 1999) or because of increased P nutrition in mycorrhizal plants (Andrade et al. 2004). Accordingly, the AMF colonization is considered extremely beneficial to the development of root nodules (Barea et al. 2005).

So far the role of AM fungi in legume improvement under metal-enriched soil is concerned, Heggo et al. (1990) have investigated the effects of AM fungi on metal uptake by soybean (Merr. "Essex"). Soils with various heavy metal contents were collected from areas in close proximity to a Zn smelter in operation for nearly 100 years. One treatment was inoculated with mixed AM fungi (600 spores per pot) and soil bacteria. The second treatment was inoculated only with soil bacteria, and the third treatment remained sterile. Soybeans were sown into each soil. After 6 weeks of growth, the plants were harvested, and the dry weight and the content of N, P, Zn, Cd, Cu, Mn, and Fe were determined in plant leaves. The amount of AM fungal colonization, nitrogenase activity, and number and weight of nodules were also determined on plant roots. Results indicate that inoculation with soil bacteria and AM fungi increased plant dry biomass and foliage P and N contents. Inoculation with AM fungi substantially reduced Zn, Cd, and Mn concentrations in leaves of soybean grown in metal-treated soil. The colonization of roots by AM fungi was reduced at the highest soil metal concentrations. These results indicate that the effect of AM fungi in heavy metal uptake is dependent upon the initial soil metal concentration. In addition, effectiveness of autochthonous bacterium and mycorrhizal fungus on *Trifolium* growth, symbiotic development, and soil enzymatic activities in Zn-contaminated soil was investigated (Vivas et al. 2006). Zinc-adapted and Zinc-nonadapted *G. mosseae* strains protected the host plant from the toxicity of Zn (600 μg g^{-1}). Zn-adapted bacteria increased root growth and N and P nutrition in plants colonized by *G. mosseae*, while it decreased the specific absorption rate (SAR) of Cd, Cu, Mo, or Fe in plants colonized by Zn-nonadapted *G. mosseae*. Symbiotic characters like nodule number and extraradical mycelium were optimum in plants colonized by Zn-adapted isolates, the most effective in increasing plant Zn tolerance. The bacterium also increased the quantity and quality (metabolic characteristics) of mycorrhizal colonization, with the highest improvement for arbuscular vitality and activity. Inocula also enhanced soil enzymatic activities (dehydrogenase, β-glucosidase, and phosphatase) and IAA synthesis, particularly in the rhizosphere of plants inoculated with Zn-adapted isolates. The findings collectively show that *G. mosseae* strains possess different inherent potential for improving plant growth and nutrition in Zn-contaminated soil. The bacterium increased the potential of mycorrhizal mycelium as inoculum. Mycorrhizal performance, particularly that of the autochthonous strain, was increased by the bacterium and both synergistically contributed to better

plant growth and establishment in Zn-contaminated soils. The investigation suggests that remediation of heavy metal-contaminated sites might be facilitated by selection of tolerant plant species. The biomasses of legumes and root nodules developed were increased by AMF colonization, possibly due to the fact that the microbial cooperation in the rhizosphere could increase the resistance of the host plant to the toxicity of heavy metals.

In other study, *G. fasciculatum*- and *Rhizobium*-inoculated *Prosopis juliflora*, grown in soils treated with tannery effluent, had higher dry weight; increased rate of photosynthesis; higher protein content; increased sugars, lipid, and amino acid levels; increased activity of enzymes catalase, peroxidase, phosphatases, and nitrate reductase; increased level of growth hormones IAA, gibberellin, and cytokinins; and higher N, P, K and Ca, Fe, Co, and Mo levels in root and shoot than their single inoculations (Selvaraj 1998). Moreover, the translocation of heavy metals such as Cd, Cr, and Zn was highly restricted by the extraradical hyphae of AM fungi, in dual culture treatments, when compared with the single inoculations. Thus, it was concluded that the composite application of AM fungi and *Rhizobium* could be more effective compared with single culture application. In a follow-up study, Al-Garni (2006) tested the efficacy of mixed culture of AM fungus and *Rhizobium* using cowpea (*Vigna sinensis*) as a test crop, grown in soils amended with varying concentrations of Zn and Cd. The microsymbionts notably increased dry weight, root/shoot ratios, leaf number and area, plant length, leaf pigments, total carbohydrates, and N and P content of infected plants as compared with noninfected controls at all levels of heavy metal concentrations. Tolerance index of cowpea plants was more increased in the presence of microsymbionts than in their absence in polluted soil. Microsymbionts dependencies of cowpea plants tended to be increased at higher levels of Zn and Cd in polluted soil. Metals accumulated by microsymbionts-infected cowpea plant were mostly distributed in root tissues, suggesting that an exclusion strategy for metal tolerance widely exists in them. This study provides evidence for benefits of rhizobia to AM fungi in the protection of host plants against the toxic effects of metals. Thus, bacterial–AM–legume tripartite pairing could be a new option to increase the heavy metal tolerance among legumes when grown in heavy metal-stressed soil.

13.7 Factors Affecting AMF in Metal-Polluted Sites

Arbuscular mycorrhizal fungi are one of the most important soil microorganisms. They expand the interface between plants and the soil environment and contribute to uptake of nutrients especially P (Li et al. 1991a). They also facilitate the uptake of N (Ames et al. 1983), Cu (Li et al. 1991a), and Zn (Bürkert and Robson 1994). The healthy plants in turn show greater tolerance to heavy metal toxicity, as reported by numerous workers (Meharg 2003). However, the exact mechanism as to how mycorrhizal fungi reduce metal toxicity is poorly understood Thus, metal uptake by plants treated with AMF can vary dramatically as a function of the particular strains and ecotypes of AMF (del Val et al. 1999b; Malcova et al.

2003) utilized, the types and ecotypes of plant (Díaz et al. 1996), and the metal (Gamalero et al. 2009) and its availability (El-Kherbawy et al. 1989). Other factors that affect the AM abundance in soils include soil edaphic conditions, including soil fertility (Lambert and Weidensaul 1991), and plant growth conditions, such as light intensity (Weissenhorn et al. 1995) or root density (Joner and Leyval 2001). In a study, Cicatelli et al. (2010) have demonstrated that suitable plant–fungus combinations may contribute to the success of phytoremediation of heavy metal-polluted soil. Regarding the negative effect of HMs on AMF, Weissenhorn et al. (1993, 1994) have reported that heavy metals reduce AM spore germination and hyphal extension *in vitro* or completely eliminate AM colonization of plant roots. On the contrary, mycorrhizal colonization efficiency of roots in heavy metal-polluted sites can invariably be affected by different metals. For example, Cd did not affect the extent of colonization in three different pea (*Pisum sativum*) genotypes (Rivera-Becerril et al. 2002), Cu did not affect colonization in poplar (Todeschini et al. 2007), and As did not affect colonization in *Pteris vittata* (brake fern) colonized by *G. mosseae* and *Gigaspora margarita* (Trotta et al. 2006). However, mycorrhizal rather than nonhost plants could still colonize polluted mining sites (Shetty et al. 1994), suggesting that heavy metal tolerance or other beneficial effects were conferred by mycorrhizal symbiosis.

13.8   Mechanisms of Heavy Metal Tolerance

The interaction mechanisms between AMF and metals, and the cellular and molecular mechanisms conferring tolerance to AMF, are poorly understood (Colpaert and Vandenkoornhuyse 2001) and, therefore, need further investigations. However, several biological and physical mechanisms have been proposed to explain metal tolerance among AM fungi and their role in protecting plants from metal toxicity.

For example, Giasson et al. (2008) claimed that the evolution of metal tolerance is rapid in mycorrhizal fungi. AM fungi have evolved an array of strategies that could alleviate heavy metal threats in the polluted agroecosystems (Kramer 2005). Further, Giasson et al. (2008) have suggested that AMF may adopt one or combination of the following mechanisms to tolerate and to resist metal toxicity: (1) fungal gene expression, (2) extracellular metal sequestration and precipitation, (3) production of metallothioneins (metal-binding proteins), (4) avoidance of metals (reduced uptake or increased efflux, formation of complexes outside cells, release of organic acids, siderophores, etc.), (5) intracellular chelation (synthesis of ligands such as poly-phosphates and metallothioneins), (6) compartmentation within leaf vacuoles, (7) loss of leaves during dry or cold seasons, (8) phosphorus plant status or interaction between P and metals (increased P uptake by host plant), (9) biological sorption via glomalin, and (10) volatilization.

The expression of several protein-encoding genes actively involved in heavy metal tolerance varies in their response to different metals. Metal transporters and plant-encoded transporters (proteins) are involved in the tolerance and uptake of metals (Hildebrandt et al. 2007) from extracellular media or in their mobilization

from intracellular stores (Gaither and Eide 2001). Göhre and Paszkowski (2006) hypothesized that metals could be released at the pre-arbuscular interface and then taken up by plant-encoded transporters. Such proteins included a Zn transporter, a metallothionein, a 90-kDa heat shock protein (hsp), and a glutathione *S*-transferase (all assignments of protein function are putative; Giasson et al. 2008). For instance, metallothionein-like polypeptides are reported to be involved in Cd and Cu detoxification by AM fungal cells since these polypeptides bind and sequester the toxic metals (Lanfranco et al. 2002). Very recently, Garg and Aggarwal (2011) have evaluated the role of AM fungus *G. mosseae* in the alleviation of Cd and Pb toxicities in pigeon pea [*Cajanus cajan* (L.) Millsp.] genotypes. The effects of interactions between Cd (25 and 50 mg kg^{-1}) and Pb (500 and 800 mg kg^{-1}) on plant dry mass, N, and production of phytochelatins (PCs) and glutathione (GSH) were monitored with and without AM fungus in genotypes Sel-85N (relatively tolerant) and Sel-141-97 (sensitive). Cadmium treatments were more toxic than Pb, and their combinations led to the synergistic inhibitions of growth and N_2-fixing potential (acetylene reduction activity [ARA]) in both genotypes. However, the effects were less deleterious in Sel-85N than in Sel-141-97. Exposure to Cd and Pb significantly increased the levels of PCs in a concentration- and genotype-dependent manner, which could be directly correlated with the intensity of mycorrhizal infection (MI). Stimulation of GSH production was observed under Cd treatments, although no obvious effects on GSH levels were observed under Pb treatments. The metal contents (Cd, Pb) were higher in roots and nodules when compared with that in shoots, which were significantly reduced in the presence of AM fungi. The results indicated that PCs and GSH might function as potential biomarkers for metal toxicity, and microbial inoculations showed bioremediation potential by helping legume plants to grow in multimetal-contaminated soils. Similar reduction in Cd translocation from roots to shoots in the presence of AM fungi is reported in roots of *Trifolium subterraneum* (Schüepp et al. 1987; Joner and Leyval 1997).

The accumulation of heavy metals in the fungal structures may represent a biological barrier. Immobilization of metals in the fungal biomass is one such mechanism involved (Zhu et al. 2001). Arbuscular mycorrhizae have often been reported to sequester and to accumulate metals in their biomass as well as in the roots of host plants. A greater volume of metals can also be stored in the mycorrhizal structures in the root and in spores. For example, concentrations of over 1,200 mg kg^{-1} of Zn have been reported in fungal tissues of *G. mosseae* and over 600 mg kg^{-1} in *G. versiforme* (Chen et al. 2001). Reduced transfer, as indicated by enhanced root/shoot Cd ratios in AM plants, has been suggested as a barrier in metal transport (Joner et al. 2000a, b). This may occur due to intracellular precipitation of metallic cations with PO^{4-}. Joner and Leyval (1997) reported that extraradical hyphae of AM fungus *G. mosseae* can transport Cd from soil to subterranean clover plants growing in compartmented pots, but that transfer from fungus to plant is restricted due to fungal immobilization. Furthermore, hyphae of mycorrhizal fungi have the ability to bind metals present in roots or in the rhizosphere which in turn results in decreased metal translocation from roots to aerial

organ (Wasserman et al. 1987). In a similar study, Turnau et al. (1993) have also demonstrated a greater accumulation of Cd, Ti, and Ba in fungal structures than in the cells of the host plant. The high sorption capacity of AM fungal extraradical mycelium for Cd, Zn (Joner et al. 2000a, b), and Cu (Gonzalez-Chavez et al. 2002) indicates that the fungal mycelium may significantly affect plant interactions with the metal ions. Uptake of metals by fungal hyphae may be influenced by chitin, an important metal-binding hyphal protein (Zhou 1999). To validate this further, Galli et al. (1994) reported that most of the metals could be bound to the cell wall components like chitin, cellulose, cellulose derivatives, and melanins of ecto- and endomycorrhizal fungi. There is also a report that fungal vesicles may play an important role in metal detoxification (Göhre and Paszkowski 2006). In maize for example, heavy metals are selectively retained in the inner parenchymal cells coinciding with fungal structures (Kaldorf et al. 1999). Furthermore, mycorrhizal fungi can bind heavy metals beyond the rhizosphere by releasing an insoluble glycoprotein commonly known as glomalin (Gonzalez-Chavez et al. 2004). Gonzalez-Chavez et al. (2004) reported that 1 g of glomalin could extract up to 4.3 mg Cu, 0.08 mg Cd, and 1.12 mg Pb from polluted soils.

Mycorrhizal fungi may also indirectly provide resistance to plants (Cardoso and Kuyper 2006). For instance, AM fungi can increase plant establishment and growth despite high levels of soil heavy metals (Enkhtuya et al. 2000), due to better P nutrition (Feng et al. 2003), water availability (Auge 2001), and soil aggregation properties (Rillig and Steinberg 2002) associated with this symbiosis. External mycelium of AM fungi provides a wider exploration of soil volumes by spreading beyond the root exploration zone (Khan et al. 2000; Malcova et al. 2003), thus providing access to greater volume of heavy metals present in the rhizosphere. Likewise, AMF also play a significant role in the improvement of rhizosphere composition (Azcón-Aguilar et al. 2003; Medina et al. 2003). AM fungi can penetrate deeper in soil and have the ability to access contaminants contained within rhizosphere (Hutchinson et al. 2003). Thus, the AM fungi can influence pH (Li et al. 1991b), microbial communities (Olsson et al. 1998), and root-exudation patterns (Laheurte et al. 1990), which together have been suggested as indirect mechanisms that could alleviate the adverse impact of soil toxicants. In this context, Göhre and Paszkowski (2006) have suggested that different mechanisms are adopted both by plants and AMF to circumvent the metal toxicity.

Conclusion

Heavy metals are a unique class of toxicants because they cannot be destructed into nontoxic forms. The consequential impact of these toxicants on environment has therefore become a global threat. High concentrations of heavy metals in soil have detrimental effects on agroecosystems and pose a serious risk to human health also as they can enter the food chain via agricultural products or contaminated drinking water. Additionally, leguminous plants have shown poor biomass production, photosynthetic activity, nitrogen fixation and nodulation, and some other physiological disorder, when grown in metal-enriched soil. Therefore, it is imperative to find ways as to how the toxicity of metals to

legumes could be reduced. In this chapter, bioremediation, a sustainable and inexpensive technology, has provided an alternative to existing physicochemical methods used for abating metal toxicity.

Among the biological materials used to remediate metal-contaminated environment, AM associations have been found quite impressive for natural and managed ecosystems primarily due to their nutritional and nonnutritional benefits extended to their symbiotic partners. Mycorrhizal associations increase the absorptive surface area which in turn enhances water and mineral uptake ability of plants. The protection and enhanced capability of mineral uptake result in greater biomass production, a prerequisite for successful remediation. AM fungi can also act as a filtration barrier against moving heavy metals to plant shoots. The AM fungi inhabiting heavy metal-polluted soils have been found as more tolerant than mycorrhizae originating from nonpolluted soils. Such tolerant mycorrhizae are reported to efficiently colonize plant roots, growing in heavy metal-stressed environments. Thus, it is urgently required to screen indigenous and heavy metal-tolerant mycorrhizae so that AM symbiosis can be used inexpensively to clean up large area of metal-contaminated soils. Phytoremediation strategies, such as phytoextraction, phytostabilization, and rhizofiltration, on the other hand are yet another relatively cheap and eco-friendly method employed to remediate metal-contaminated soils. It is therefore of great practical importance that one can combine selected plants with specific AM fungal cultures capable of adapting to higher concentrations of heavy metal in future research for phytoremediation programs. Besides facilitating plant growth by providing nutrients to plants, AMF have evolved diverse strategies to overcome metal toxicity. Such mechanisms may include immobilization of metal compounds, decreased metal uptake, precipitation of polyphosphate granules in the soil, adsorption to chitin in the fungal cell walls, and chelation of heavy metals inside the fungi. However, there is a need to develop new methods and to optimize conditions for mass propagation and application of AMF. The lack of correlation between mycorrhizal colonization rates and host response is perhaps one of the areas that requires considerable attention.

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