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Dharmendra Kumar Gupta *Editor*

Plant-Based Remediation Processes

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Editor

Plant-Based Remediation Processes

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This book is dedicated to my beloved mother
Late Smt. Annapurna Gupta
1949–2011

Preface

The idea of cleaning up contaminated environments by using green plants is not new. About 300 years ago, plants were proposed to be used in the treatment of wastewater (Hartman 1975). At the end of the nineteenth century, *Thlaspi caerulescens* and *Viola calaminaria* were the first plant species documented to accumulate high levels of metals in leaves (Baumann 1885). At present, there are about 420 species belonging to about 45 plant families which have been reported as hyperaccumulators of heavy metals (Cobbett 2003). Although the identification of new plant species with this property is still growing from field collections (Krämer 2003), only a few species have been tested in the laboratory to confirm their hyperaccumulating behaviors. The urgency to discover hyperaccumulators has shown several intriguing patterns (Baker and Whiting 2002). First, several plant families contain an inexplicably high number of hyperaccumulators: among those are Asteraceae, Brassicaceae, Euphorbiaceae, Fabaceae, Flacourtiaceae, and Violaceae, suggesting that several families and genera within them may be pre-adapted/predisposed to deal with high concentrations of metal. Second, there appears to be a disproportionately high percentage of hyperaccumulators in tropical regions.

Plant tolerance to heavy metals depends largely on plant efficiency in the uptake, translocation, and further sequestration of heavy metals in specialized tissues or in trichomes and organelles such as vacuoles. The uptake of metals depends on their bioavailability, and plants have evolved mechanisms to make micronutrients bio-available. Some plants have developed resistance to high metal concentrations, basically by two mechanisms, avoidance and tolerance. The first mechanism involved exclusion of metals outside the roots, and the second mechanism consists basically in complexing the metals to avoid protein and enzyme inactivation. Some plants can also accumulate metals in their tissues at concentrations higher than those found in the soil, and these plants as referred as hyperaccumulators (Gupta and Sandalio 2012).

Given the nature and extent of contamination worldwide and the costs involved in remediation, recent years have seen a drive toward alternative yet effective technologies for the remediation of polluted sites. In this regard, bioremediation,

typically referring to microbe-based cleanup, and phytoremediation, or plant-based cleanup, have generated much interest as effective low-cost and environmentally friendly technologies for the cleanup of a broad spectrum of hazardous organic and inorganic pollutants (Pilon-Smits 2005). Plant-based environmental remediation has been widely pursued by academic and industrial scientists as a favorable low-impact cleanup technology applicable in both developed and developing nations (Robinson et al. 2003). Physiological, biochemical, and molecular approaches are continually being applied to identify the underlying mechanisms of metal tolerance and hyperaccumulation (Lasat 2002). The drive to find genes underlying these unique biological properties is partly fueled by interest in using transgenic plants in phytoremediation (Pilon-Smits 2005). Interestingly, as transgenics are being tested in the field and the associated risks assessed, their use appears to be more accepted and less regulated than has been the case for transgenic crops (Pilon-Smits and Pilon 2002).

In last two decades phytoremediation work got so much attention from the scientists and researchers throughout the globe. The main purpose of this book is to present recent advances in the field, mainly on the use of green plants for remediation of various metal/metalloids. Other key features of the book are related to biomonitoring of heavy metal pollution, different amendments for higher uptake of toxic metals, transport of heavy metal in plants, mechanism of toxicity, and remediation through engineering plants. Some chapters are also dealt with transgenic as well as metallomics approaches for the remediation of heavy metal/metalloids. Some chapters are focusing on recent protocols for phytotechnological tools for metal contaminations. Overall the information compiled in this book will bring in-depth knowledge and advancement of phytoremediation technologies in recent years.

Dr. Dharmendra Kumar Gupta is personally thankful to the authors for contributing their time, knowledge, and enthusiasm to bring this book into shape.

Mol, Belgium Dr. Dharmendra Kumar Gupta

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Contents

Chapter 1
Phytoremediation Protocols: An Overview

Soumya Chatterjee, Anindita Mitra, Sibnarayan Datta, and Vijay Veer

1.1 Phytoremediation: An Introduction

Growth and development of any organism is always influenced by the environment. It is axiomatic that, plants do have unique characteristics to deal with wide-ranging of ambience that involve different fluctuating conditions like climate, temperature, moisture, and soil conditions (Norman 1962). Along with water, nutrients, and minerals essential for their growth, plants take up a diversity of natural and noxious compounds through their root system from soil and ground water. To survive with all such essential and nonessential components, plants use to develop diverse detoxification mechanisms within their system (Singer 2006). Microorganisms present in the rhizosphere region of plants have the ability to eliminate several contaminants from the surroundings by a range of enzymatic processes. Consequence with of their versatility, adaptability, and diversity in the environment, a number of microorganisms along with plants may be regarded as the excellent system to remediate most of the environmental contaminants, including organic and inorganic contaminants ones (Lovley 2003). Keeping in view of these attributes, plants may be regarded fundamentally as a "natural, solar powered pump and treat system" (Pilon-Smits 2005) for cleaning of contaminated sites leading to the concept of phytoremediation, a natural, esthetically pleasing, and low cost technology.

Phytoremediation (Ancient Greek: *phyto*-"plant," and Latin *remedium*-"restoring balance") describes the treatment of diverse environmental pollution problems. According to the recent definition presented by Landmeyer (2011), phytoremediation is the "application of plant-controlled interactions with groundwater and organic and

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inorganic molecules at contaminated sites to achieve site-specific remedial goals." Cleaning up of the environment through plants are rendered by direct uptake of the toxic chemical, followed by subsequent transformation, transport, and their accumulation in less toxic forms (Schnoor et al. 1995). In addition, plants support remediation process by releasing exudates and enzymes that induce microbial diversity at rhizosphere and biochemical activity in the bulk soil and mineralization (Macek et al. 2000).

Phytoremediation techniques are developing great interest because the method became an alternative to the conventional energy intensive, instrument, and chemical-based expensive restoration technologies of vast polluted areas of land and water (Azadpour and Matthews 1996; Garbisu et al. 2002; Vassilev et al. 2004; Padmavathiamma and Li 2007; Lone et al. 2008) and thus decontaminating the polluted environment by improving the utility, even of the marginal lands (Meagher 2000). The concept of cleaning pollutants using green living systems for environmental remediation is quite old. Nickel accumulation by the plant *Alyssum bertolonii* was first reported in 1948; however, the concept received momentum after the reports from the researcher Robert Brooks, of Massey University in New Zealand in 1977. Thereafter, widespread researches on the use of wetland plants, for treating heavy metals, radionuclide contaminated waters were initiated. After the nuclear disaster at Chernobyl, Ukraine, in 1986 *Phytotech* began using plants to decontaminate water and soil. This was to be proving ground for new technology. Iowa City used tree farms to clean landfills in 1989, after the results published from Phytotech experiments. In 1990, nitrogen-rich aquifer in New Jersey was managed by phytoremediation technology. The first *Living Machine* was designed and constructed in Europe during 1995, which lead to researching genetic engineering applications. Research proved that specific plants were capable of removing toxins and certain metals. The Department of Defense and EPA joined forces to develop plant-based cleanup approaches to large-scale cleanup projects (Rai and Pal 1999).

Phytoremediation of toxic elements like mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), cesium (Cs), and strontium (Sr) involves extraction and translocation of toxic cation or oxyanion to above ground tissues by plants for later harvest, converting the element to a less toxic chemical species (Meagher 2000). On the other hand, for organic pollutants, such as polychlorinated biphenyl (PCBs), dioxin, polycyclic aromatic hydrocarbons (PAH), trichloroethylene, the target of phytoremediation is to completely mineralize them into relatively nontoxic constituents, such as CO_2, nitrate, chlorine, and ammonia (Cunningham et al. 1996). Plants have several strategies (Fig. 1.1) for dealing with xenobiotics: phytostabilization, phytoextraction, phytovolatilization, rhizofiltration, phytodegradation, and phytostimulation (Salt et al. 1998; Fulekar et al. 2009; Marques et al. 2009). For soil phytoremediation, phytostabilization and phytoextraction are preferred (Salt et al. 1998).

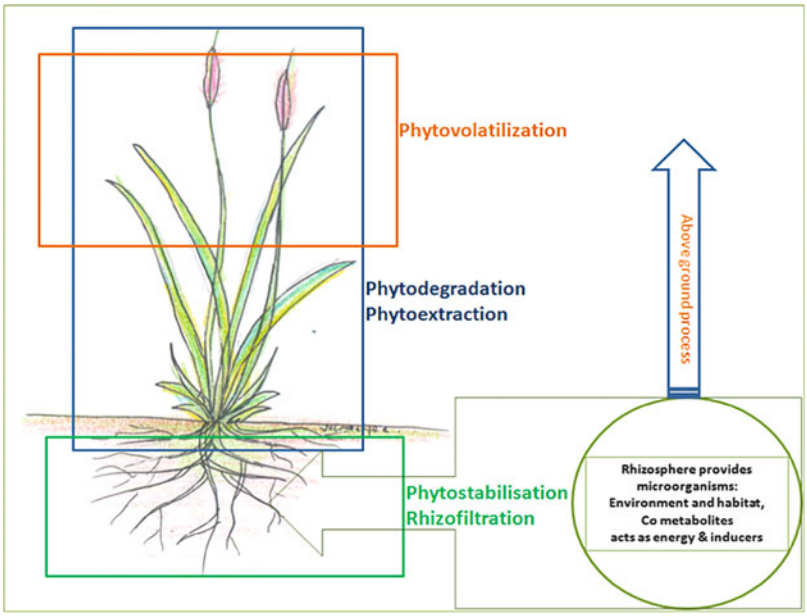

Fig. 1.1 Major processes of phytoremediation where root zone (rhizosphere) plays an important role in contaminant uptake and stabilization

1.2 Phytostabilization: Mobility Reduction of Contaminants

Phytostabilization is the process to reduce the mobility of contaminants in soil through adsorption onto roots, adsorption and accumulation by roots, or precipitation within the root zone. Vegetation are used to provide stabilization of migration of contaminants by leaching, erosion, or dispersion along with soil, water, or air to prevent pollution to ground water and surrounding environments (Ernst 2005). Plants suitable for phytostabilization should develop an extensive root system that provide good soil colonization, possess tolerance to the contaminant metals, ideally immobilize the contaminants in the rhizosphere (Kramer 2005), and endure drought and high temperature as well (Ernst 2005). This technique generally employs metal-tolerant varieties of grass species such as *Agrostis capillaris* and *Festuca rubra* (Kidd et al. 2009) but the leguminous species *Lupinus albus* also has been suggested as a good candidate for remediation of Cd and As-contaminated soil (Vazquez et al. 2006).

In addition, soil amendments are indispensable to achieve a long-term phytostabilization such as (1) increasing soil pH to more than 5 by liming with $CaCO_3$ and/or $Ca(OH)_2$ (Mench et al. 1994), (2) immobilization of heavy metals by the application of soil additives such as compost (Vangronsveld et al. 1995), and (3) improving soil quality by fertilization (Li and Chaney 1998). The toxic elements, chiefly chromium and lead can be promisingly phytostabilized. Deep-rooted plants

effectively reduce the highly toxic and soluble Cr^{6+} compounds to insoluble Cr^{3+}, which does not pose an environmental risk (James 1996). Chemical species of Pb in soil are usually somewhat bioavailable, whereas, chloropyromorphite, a Pb phosphate mineral is both extremely insoluble and non-bioavailable (Ma et al. 1995). The roots of *Agrostis capillaris* growing in highly contaminated Pb/Zn mine wastes are known to form pyromorphite from soil lead and phosphate by an unknown mechanism, thus minimizing the escape of lead movement (Cotter-Howells and Capom 1996). Advantage of using grass species for phytostabilization is that they bioaccumulate less metals in their shoots in comparison to dicot species, in this way minimizing exposure of wildlife to toxic elements (Pilon-Smits 2005).

1.3 Phytoextraction

Phytoextraction involves the cultivation of higher plants that concentrate and translocate soil contaminants in their above ground tissues that can be harvested at the end of the growth period (Salt et al. 1998). It is the most effective among several phytoremediation methods, although technical difficulties are there for its applications (Kramer 2005). Selection of suitable plant species is crucial for effective phytoextraction and biomass derived from shoot of a phytoremediator crop plant should be capable of depositing metal(oid) species at concentration 50–500 times higher than those in the contaminated soil substrate (Kramer 2005). The best-known natural hyperaccumulators plants are alpine pennycress (*Thlaspi caerulescens* L.) capable of hyperaccumulating Zn^{2+}, and occasionally Cd^{2+} and Ni^{2+} (Milner and Kochian 2008), the serpentine endemic shrub *Alyssum* sp., Indian mustard *Brassica juncea* (Brassicacea) and *Astragalus racemosus* (Leguminosae). The Asian stonecrop *Sedum alfredii* (Crassulaceae) has gained increased attention due to higher accumulation rate of Zn, Cd, and Pb (Lu et al. 2008; Deng et al. 2008). Plants ideal for phytoextraction besides having an inherent capacity to tolerate and hyperaccumulate metals should possess multiple traits like (1) high and fast growing biomass; (2) extensively branched root systems; (3) ability to grow outside their area of collection; (4) relatively easy to cultivate; and (5) possible repulsive to herbivores to avoid the escape of accumulated metals to the food chain (Seth 2012). Unfortunately, most of the naturally hyperaccumulating plants have slow growth, poor biomass, and often strong association with a specific habitat, therefore limiting the phytoextraction potential (Chaney et al. 2005). However, non-hyperaccumulator plants having higher growth rate and biomass could be modified or engineered to achieve the above-mentioned attributes. To increase the potential of phytoextraction, factors limiting trace element accumulation in plants have to be resolved, which may include mobilization of poorly available contaminant in the soil, root uptake, sequestration by metal-complex formation and deposition in vacuoles for detoxification within roots, translocation to symplast, efficient xylem loading, distribution and storage inside the aboveground organ and tissues, and eventually expulsion of accumulated metal to less metabolically active cells, e.g., trichomes (Clemens et al. 2002). Two approaches are currently being explored to

improve or modify the metal accumulating plants: the conventional breeding and genetic engineering. Although a number of reports exist on successful crop breeding (Gleba et al. 1999; Dushenkov et al. 2002; Alkorta et al. 2004; Nehnevajova et al. 2007) yielding improved metal accumulator plants, the major constraint in developing such hybrid is sexual incompatibility between the taxa. Transgenic plants have opened new avenues in phytoremediation technology by expressing the desired gene and overcoming the limitations imposed by sexual incompatibility.

1.3.1 Transgenic Approaches to Develop Metal-Accumulating Plants

Metallophytes have distinct biological mechanisms that enable them to tolerate high tissue metal concentration. Recent progress in understanding the molecular basis of metal accumulation and tolerance by metallophytes has provided a strong scientific basis for creating transgenics that enhance phytoextraction potential. Some of the possible areas of genetic manipulation are outlined below:

- Metallothioneins (MT) and phytochelatins (PCs) are known as metal-chelating proteins, responsible for the detoxification and accumulation of metals (Hirata et al. 2005). Genetic manipulation of the plants for synthesis of metal chelators will improve the capability of plants for metal uptake by increasing the availability of such metals (Pilon-Smits and Pilon 2002; Clemens et al. 2002; Lee et al. 2003).
- Genes involved in metal uptake, translocation, and sequestration in plants are well studied. Introduction or overexpression of any of these genes into candidate plants (Table 1.1) could be a way to enhance the previously mentioned pathway in non-hyperaccumulators (Clemens et al. 2002). Transgenic plants overexpressing the genes encoding the enzymes for histidine biosynthesis and ACC deaminase, Hg^2-reductase, glutathione synthetase, arsenate reductase, and aldolase/aldehyde reductase, were shown to become more tolerant to the toxic levels of metals and carried out phytoextraction with increasing potential (Stearns et al. 2005; Thomas et al. 2003; Bennett et al. 2003; Shah and Nongkynrih 2007).
- The repression of an endogenous gene expression by inserting an antisense RNA can also result in enhanced metal uptake by plants (Shah and Nongkynrih 2007).
- The introduction of an additional metal-binding domain to the implemented protein further enhances the metal-binding capacity (Kotrba et al. 1999).
- Another promising approach is overexpressing the enzymes catalyzing rate-limiting steps. ATP sulfurylase (APS) is such a rate-limiting enzyme in the selenium detoxification processes. The overexpression of APS in transgenic *Brassica juncea* led to three times more uptake and accumulation of selenium in comparison to wild plants (Pilon-Smits et al. 1999).

Table 1.1 Selected examples of genetically engineered plants with the respective gene transferred, gene product, gene source, and improved trait

Gene	Product	Source	Target plant species	Observed effect	References
MT1A	Metallothionein	Mouse	*Nicotiana tabacum*	Cd tolerance	Pan et al. (1994)
TaPCS	Phytochelatin synthase	Wheat	*Nicotiana glauca*	Pb accumulation	Gisbert et al. (2003)
APs	ATP-sulfurylase	*Arabidopsis thaliana*	*Brassica juncea*	Se hyperaccumulation	Banuelos et al. (2005)
merA18	Hg(II) reductase	Gram − ve bacteria	*Liriodendron tulipifera*	Hg tolerance and volatilization	Rugh et al. (1998)
merB	Organomercurial lyase	Gram − ve bacteria	*Arabidopsis thaliana*	Hg tolerance and volatilization	Rugh et al. (1996)
arsC	Arsenate reductase	*Escherichia coli*	*Brassica juncea*	As tolerance	Dhankher et al. (2002)
GSH1	Glutathione synthetase	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*	Increased accumulation of Cd and As	Guo et al. (2008)

- Another strategy for increasing the efficiency of phytoextraction involves increase in the metal translocation to shoots by increasing plant transpiration (Gleba et al. 1998).
- According to Raskin (1996), transgenic plants could be developed to secrete metal selective ligands (phytosiderophores or chelating agents) into the rhizosphere, which could specifically solubilize the toxic elements (Ma and Nomoto 1996).

1.3.2 Phytoextraction with Endophytic Microbes

Researchers carried out several experiments on the application of endophytic bacteria and mycorrhizal fungi in the phytoextraction of pollutants (Doty 2008). Endophytes are the symbiotic microbes inhabiting in the internal plant tissue and are able to facilitate plant growth and increase resistance of plants against pathogen and drought (Taghavi et al. 2010). It has been recently reported that the endophytic symbiotic bacteria *Methylbacterium populum* that lives within poplar can mineralize 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (VanAken 2009). However, the success rate of phytoextraction of heavy metals using endophytic bacteria remains slow because of the lack of proper strains with heavy metal resistance and detoxification capacities (Luo et al. 2011). Besides endophytes, the arbuscular mycorrhizal (AM) fungi are also known to be involved in the uptake of elements into plants (Doty 2008) and are reported to be present in mutualistic association in the roots of plants growing on markedly contaminated soil (Khade and Adholeya 2009; Javaid 2011; Miransari 2011). Therefore, mycorrhizal fungi can be applied for significant phytoextraction by improving several attributes like increased metal tolerance, increased biomass production, and greater metal concentration in plant tissue (Vamerali et al. 2010). In brief, the goal of phytoextraction is to reduce the presence of trace elements in soils through their uptake and accumulation by plants; in contrast, phytostabilization aims to minimize the mobile and bioavailable fraction of metals by combining the use of metal-tolerant plants and soil amendments and thus reduces leaching through soil. In both processes the "mobility and bioavailability of trace elements in the soil—particularly in the rhizosphere where root uptake and exclusion takes place—is a critical factor affecting their outcome and success" (Kidd et al. 2009).

1.4 Phytovolatilization

A variant of phytoextraction is phytovolatilization, where the contaminant is not primarily concentrated in aboveground tissues, but instead transformed by the plant into evaporable and less toxic form before releasing into the atmosphere (Kramer 2005). It is not a direct clean up method rather a dispersal technology of the

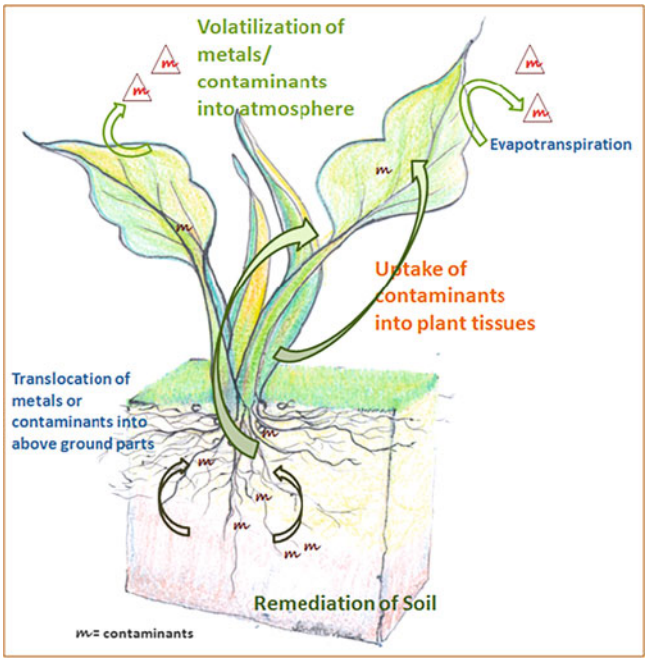

Fig. 1.2 Schematic representation of phytovolatilization where metals are volatilized by the process of evapotranspiration by plants

contaminants. Phytovolatilization is very much promising for mercury (Hg) and selenium (Se) in which metals are converted to a volatile form for release and dilution into the atmosphere (Bhargava et al. 2012). This method is advantageous over other phytoremediation methods as it removes metal(loid) from a site without the need of harvest/disposal of contaminated plants (Fig. 1.2).

1.4.1 Detoxification of Mercury by Plants

The most spectacular achievements of biotechnology in phytoremediation were the engineering of plants capable of removing methyl-Hg from contaminated soil (Rugh et al. 1996; Brunner et al. 2008). The purpose is achieved by the introduction of bacterial *mer*A and *mer*B genes into several plant species including *Arabidopsis*, tobacco, poplar, rice, and cottonwood (Rugh et al. 1996; Bizily et al. 2000; Heaton et al. 2003; Czako et al. 2006; Lyyra et al. 2007). The *mer*A gene encodes an NADPH-dependent mercuric ion reductase which converts Hg^{2+} to nontoxic volatile metallic Hg^{0} and *mer*B encodes organomercurial lyase liberating Hg^{2+} from organomercurial compounds $R\text{-}Hg^{+}$ (Silver and Phung 2005). Transgenic *A. thaliana* (Rugh et al. 1996; Yang et al. 2003), *Nicotiana tabacum* (Ruiz et al.

2003), *Oryza sativa* (Heaton et al. 2003), yellow poplar *L. tulipifera* (Rugh et al. 1998), overexpressing bacterial *mer*A and/or *mer*B become more tolerant to Hg^{2+} and R-Hg^{+} and release 10 times higher elemental Hg as compared to nontransformed plants. It has been reported that transgenic plants in which *MerB* is targeted in the endoplasmic reticulum rather than cytoplasm, release mercury in tenfold higher volatile form (Bizily et al. 2003).

1.4.2 Detoxification of Selenium by Plants

Two pathways dominate in the natural detoxification of selenium (Se) in plants. In most species, selenium is most toxic after metabolization into analogues of amino acid cysteine and methionine. Selenium hyperaccumulating plant species have a specific enzyme, selenocysteine methyltransferase (SMT) which is responsible for converting selenate into methyl selenocysteine (MetSeCys), ultimately incorporated into the proteins and thus resulting in hyperaccumulation of selenium. In a second detoxification mechanism, selenate can be metabolized into dimethylselenide (DMSe) which is 100 times less toxic than selenate and selenite in soil and volatilized from leaves and roots (Terry et al. 2000). Transgenic Indian mustard (*Brassica juncea* L.) transformed with the SMT gene from Se-hyperaccumulator *Astragalus bisulcatus* releases a higher DMSe in addition to an improved Se accumulation and tolerance in comparison to the control plants (LeDuc et al. 2004).

1.5 Rhizofiltration

This phytoremediation method can be defined as the use of aquatic plants, either floating or submerged to absorb, concentrate, and remove hazardous compounds particularly heavy metals or radionuclides from aqueous environment by their roots (January et al. 2008; Eapen et al. 2003) (Fig. 1.3). A suitable plant for rhizofiltration should have larger root system through which toxic metals are taken up from solution over an extended period. Such plant should be capable of producing up to 1.5 kg (dry weight) of roots per month per m^2 of water surface (Dushenkov et al. 1997). Rhizofiltration usually involves in hydroponically cultivated plants in a stationary or moving aqueous system wherein the plant roots absorb pollutants from the water (Salt et al. 1995). Candidate plant for rhizofiltration includes the Indian mustard (B*rassica juncea*), sunflower (*Helianthus annuus*), and corn (*Zea mays*) (Brooks and Robinson 1998). Success of rhizofiltration greatly depends on the physicochemical characteristics of the plants, which may favor the process of bio-adsorption (Olgu𝑛 and Sanchez-Galvan 2012).

Dushenkov et al. (1995) reported that within 24 h, submerged roots of sunflower plants were able to substantially reduce the levels of Cd, Cr, Cu, Mn, Ni, Pb, Sr, U, and Zn in water bringing metal concentration close to or below the discharge limit.

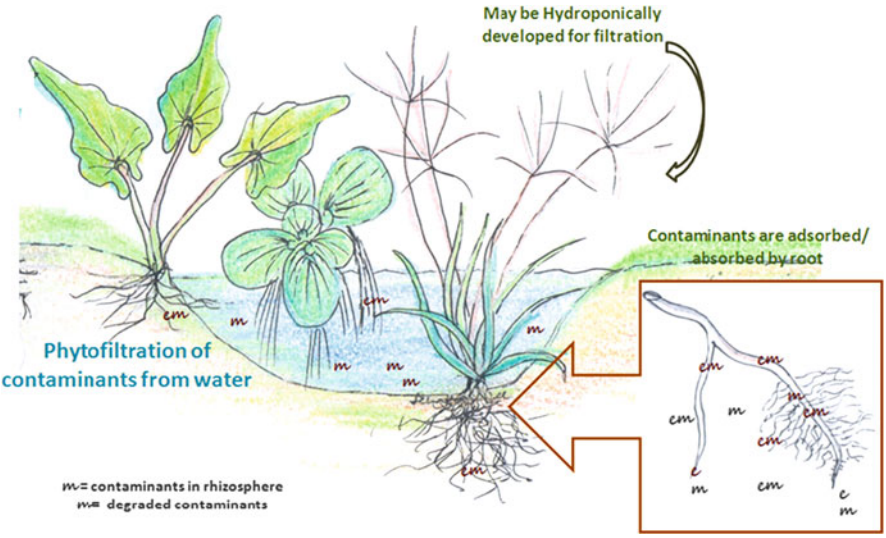

Fig. 1.3 Schematic representation of rhizofiltration where contaminants are adsorbed from water by wetland plants

Because this method is especially effective in situation involving large volume of water and relatively low concentration of contaminants, it is particularly applicable to radionuclide-contaminated water (Dushenkov et al. 1997). In a similar test carried out in Astabula, Ohio, it was found that, within 24 h, submerged roots of sunflower plants incredibly reduced the uranium level from a range of 100–400 ng mL^{-1} in contaminated water bodies to below the EPA standard level of 20 ng mL^{-1} (Cooney 1996). Several physicochemical technologies may also be executed for removal of toxic metal from wastewater such as chemical precipitation, ion exchange, adsorption, membrane filtration, photocatalytic degradation, and electrochemical method (Fu and Wang 2011). Disadvantages of these methods are high cost and disposal problem, making difficult their application in large scale. On the contrary, rhizofiltration offers a cost effective and eco-friendly alternative for the removal of contaminants from water (Rai 2012).

1.6 Phytodegradation

This method is also known as phytotransformation that refers to uptake of contaminants with the subsequent breakdown, mineralization or metabolization by plants itself through various internal enzymatic reaction and metabolic processes (Salt et al. 1998; Spaczynski et al. 2012). Subsequently many of these uptaken substances may even be metabolized into CO_2 and H_2O by enzyme complexes involved in the plant metabolic cycle (Mc Cutcheon and Schnoor 2003). The ideal

plant for use of phytodegradation should have (1) highly developed root system that has the ability to secret a considerable amount of enzyme for degradation of the xenobiotics, (2) tolerance to the xenobiotics at a concentration found in soil, (3) fast growth, and (4) a relatively high biomass (Wang and Chen 2007). The enzymes secreted from plant root into soil include laccases, dehalogenase, nitroreductase, nitrilases, and peroxidases (Carreira and Wolfe 1996; Schnoor et al. 1995; Duran and Esposito 2002; Jansen et al. 2004; Wang et al. 2004). In a field test reported by Wolfe et al. (1993), plant-derived enzymes nitroreductases and laccases showed significant degradation of TNT, dinitromonoaminotoluene, mononitrodiaminotoluene and triaminotoluene. Another study reported the degradation of various nitroaromatic compounds by nitroreductase secreted by plants (Boyajian and Carreira 1997). In another report, laccases have been shown to be useful for the degradation of a variety of persistent environmental pollutants including alkenes, bisphenol A, and synthetic dyes (Mayer and Staples 2002). The presence of plant-derived enzymes capable of degrading environmentally hazardous xenobiotics thus can be successfully exploited for the development of future phytoremediation strategies (Salt et al. 1998).

1.7 Phytostimulation

It is also called rhizospheric biodegradation and is based on the secretion by plants in root exudates which support the growth and metabolic activities of diverse fungal and bacterial communities in the rhizosphere capable of degrading varied pollutants (Anderson et al. 1994). The secreted enzymes can transform the chemicals in the rhizosphere; therefore, the plants do not need to take up the pollutants for detoxification (Fig. 1.4). Plants are able to increase the abundance of soil microflora in the rhizosphere by 1–4 orders of magnitude compared to the surrounding bulk soil and these microflora show greater range of metabolic capabilities than the microbes in the surrounding loose soil (Walton et al. 1994; Salt et al. 1998). Some plants such as mulberry (*Morus rubra*) preferentially harbor PCB degrading microbes in the rhizosphere (Wenzel et al. 1999). Rhizospheric microorganisms may also decontaminate areas by volatilizing pollutants such as polynuclear aromatic hydrocarbons (PAH) or by increasing the production of humic substances from organic pollutants (Cunningham et al. 1996; Dec and Bollag 1994).

1.7.1 Genetically Modified Plants for Improved Phytostimulation

The most promising approach of rhizospheric phytodegradation is the production of transgenic plants targeted for secreting the enzymes or factors involved in phase I and phase II detoxification process in plants (Spaczynski et al. 2012). Xenobiotics, such as PCB, various herbicides, and explosives can be successfully degraded by

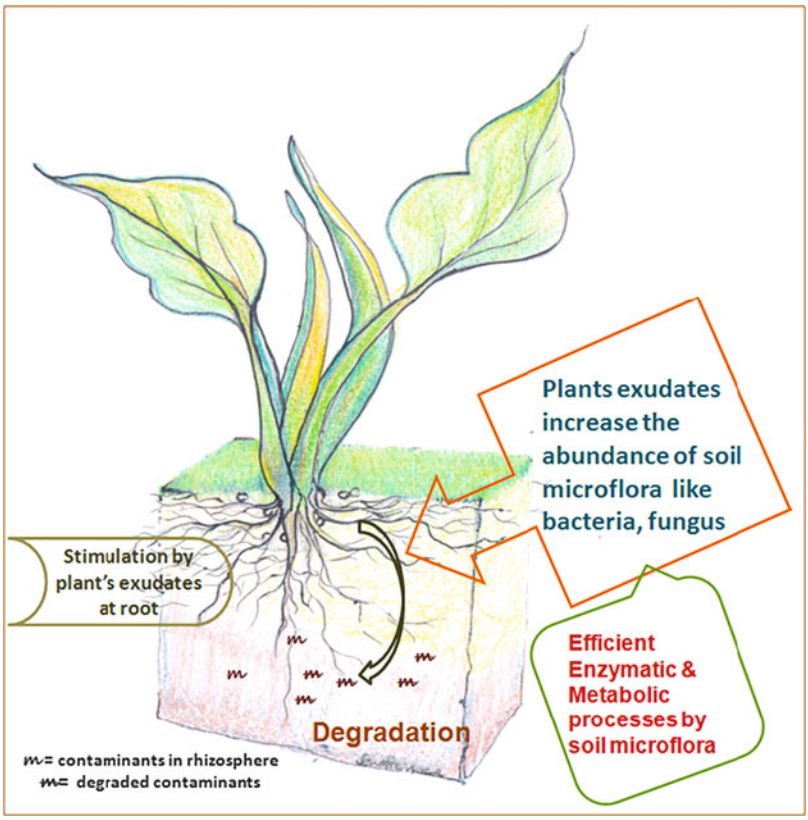

Fig. 1.4 Schematic representation of phytostimulation where plant exudates stimulate the microflora of root zone to degrade contaminants

phytostimulation. In the past decades, many successful attempts have been made with transgenic plants. Some of which are listed below:

- Mammalian cytochrome P450 gene inserted into the plants as *Nicotiana tabacum*, *Solanum tuberosum*, *Oryza sativa*, and *Arabidopsis thaliana* exhibited increased tolerance to herbicides mainly atrazine and simazine and showed a marked increase in the capability of metabolism of various xenobiotics (Doty et al. 2000; Eapen et al. 2007).
- Transgenic Indian mustard (*B. juncea*) expressing glutathione transferase (GSTs), a phase II cellular detoxification gene, shows increased tolerance to atrazine, metachlor, phenanthrene, and 1-chloro-2,4, dinitrobenzene (Flocco et al. 2004). Overexpression of GST genes enhances the potential for phytodegradation of herbicides (Kawahigashi 2009).
- Rhizodegradation of pollutant bisphenol A and PCB was efficiently carried out by transgenic tobacco plants inoculated with the gene coding laccase obtained from a fungus *Coriolus versicolor* (Sonoki et al. 2005).

- Transgenic plants are reported to remove explosives residue successfully from soil contaminated by highly toxic and mutagenic nitroglycerin, TNT, RDX, aminodinitrotoluene (Hannink et al. 2001; Rylott et al. 2006).
- *Arabidopsis thaliana* transformed with an extradiol dioxygenase gene remove 2,3- dihydroxybiphenol with high efficiency (Uchida et al. 2005).

1.8 Concluding Remarks

Phytoremediation techniques exploit the unique, selective, and naturally occurring uptake capabilities of plant root system, together with the translocation, bioaccumulation, or detoxifying abilities of the entire plant body. There are increasing number of reports suggesting that phytoremediation should become the technology of choice for remediation due to its cost efficiency and ease of implementation. Although phytoremediation techniques are successfully used in many contaminated sites in some developed countries, this technology is still in its infancy and yet to be applied commercially. In the last decades, a number of research projects have been carried out regarding production of suitable transgenic plant to increase potential phytoremediation in different countries but never has been implemented in the real contaminated sites. Restriction over field release of such genetically manipulated plants includes increased invasiveness and decreased genetic diversity of native plants due to interbreeding. Application of sterile clones may solve the problem (Abhilash et al. 2009). Another major procedural constriction is the insufficiency of knowledge regarding the specific enzyme involved in the detoxification of different pollutants by plants. Therefore, increased understanding of the enzymatic process involved in plant detoxification of diverse xenobiotics is necessary to provide information on which gene should be engineered and that will open new gateway for manipulating plant with superior remediation potential. In addition, agronomic improvement ranging from traditional crop management techniques (use of pesticides, soil amendments, fertilizer, etc.) to some precise phytoremediation approaches such as application of plants combined with microorganisms for efficient contaminant extraction (rhizoremediation) and improving metal solubility in soil by using suitable chelating agents is suggested for significant progress of phytoremediation capabilities.

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Chapter 2
Protocols for Applying Phytotechnologies in Metal-Contaminated Soils

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2.1 Introduction

Phytoremediation is becoming well-known word in both scientific literature and more popular publications. The word itself is derived from the Greek word *phyto*s (plant) and the Latin word *remedium* (roughly translated as restoration of balance/equilibrium). This makes phytoremediation a very broadly applied expression: in fact, it can be defined as any use of plants to restore the quality of soil, biota, water, and air (McCutcheon and Schnoor 2003; McCutcheon and Jørgensen 2008). Phytoremediation is considered the only solution which approaches the problem from an eco-sustainable point of view: environmentally friendly and relatively cheap. The United Nations Environment Program (2003) promotes its application as sustainable technology to remediate environmental pollution. Moreover, the European Union regulators proposed within the Directive 2008/1/EC a guideline to select the most suitable technique according to criteria such as environmental friendliness, preexisting scientific knowledge, or required time. Such guidelines leave stakeholders to choose the best remediation technology for their site, considering the economic, environmental, and social variables (Conesa et al. 2012). In this chapter the use of the phenomenon phytoremediation is narrowed down to heavy metals as pollutants and soils as the environmental compartment, focusing on phytoextraction (Raskin 1995; Blaylock et al. 1997) and phytostabilization (Berti and Cunningham 2000; Bolan et al. 2011). Phytoextraction aims to remove the heavy metal using specific plants, often in combination with specific soil additives,

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while phytostabilization aims to reduce the mobility/bioavailability of heavy metals in the soil and the re-vegetation of the site, often in combination with adding adsorbents and other chemicals to the soil (Kucharski et al. 2005; Mench et al. 2003). Normally technologies should be defined in detail regarding their application protocol, efficiency, and cost–benefit calculations. In the case of phytoextraction and phytostabilization, however, it is not possible to establish fixed schemes and procedures based on exact data from technology evaluations. This is limited by the nature of the technology itself which has to deal with soil complexity in relation to heavy metal biogeochemistry, plant behavior in relation to agronomic practice and climate conditions, variations in plant varieties within one species regarding uptake, phytotoxicity of heavy metals, etc. The authors of this chapter gained experience on this issue during the past 15 years, developing a realistic and balanced view on the applicability of phytoextraction and phytostabilization of heavy metals in soils. This includes awareness of the intrinsic methodic limitations and site-specificity, thus contributing to avoiding phytoremediation to become a "hype" which after unavoidable failures would possible have backfired to the approach itself. Many studies have been conducted in this field in the last two decades. Numerous plant species have been identified and tested for their traits regarding the uptake and accumulation of different heavy metals. Mechanisms of metal uptake at the whole plant level and at cellular levels have been investigated (Clemens 2006). Progress has been made in the mechanistic and practical application aspects of phytoremediation. They are briefly reviewed and reported in this chapter.

2.1.1 The Importance of a Feasibility Test

As the technology is based on site-specific variables (soil characteristics, contaminant levels, vegetation type, etc.), many variables during the implementation of a phytoremediation process make fulfilling the objectives not always easy to attain. In order to avoid that this could happen or, better, in order to minimize the likelihood that the process proves to be not corresponding with our goals at the end, it is imperative, before starting any real-life phytoremediation project, to perform checks, which together are defined as a "feasibility test" (Nowolsieska-Sas et al. 2005). In practice, a feasibility test simulates in a controlled environment the chemical, physical, and biological processes at stake and the conditions which are assumed to prevail in the field during phytoextraction or phytostabilization implementation.

A feasibility study or test is therefore an essential step to imitate as closely as possible the real situation. The test is basically carried out by sampling the soil matrix to be treated in a way as representative as possible for the whole site; the test will therefore be carried out on real samples taken from the site. The test includes all the analyses to characterize the soil and the contaminant behavior. After that, the test proceeds with the selection of the most appropriate plant, based on the soil analyses and on available literature experiences and references. This selection can include specific lysimeter or pot experiments. The results obtained from the

feasibility test can be used subsequently to decide whether it is possible or not to apply phytotechnology in the real field context, and if so, which approach gives the lowest risk of failure in attaining remediation goals (Koopmans et al. 2008a, b).

2.1.2 The Concept of Heavy Metal Bioavailability and Its Importance in Phytotechnologies

It is scientifically accepted that the risky fraction of metals are the mobile/bioavailable fractions, despite the fact that this terminology (especially regarding bioavailability) is vague and various definitions are given in the last few decades. It is well-known that during workshops attended by both soil chemists and soil biologists normally additional definitions are invented. Despite the lack of widely accepted definitions, the message is clear: the total heavy metal content in a soil gives no accurate indication regarding risks which are related to the heavy metal contamination, including phytotoxicity, leaching risks, and uptake by plants (i.e. food-chain propagation) (Barbafieri et al. 1996; Barbafieri 2000). It can be boldly stated that elimination/reduction/stabilization of the risky fractions is the most necessary and valuable action to solve the problems caused by contaminated soil. The main problem of this statement is the fact that policy makers have to convince; this might be difficult as many soil quality standards are still based on total concentrations in the soil. In this chapter, authors will focus on the description of applicability protocols for phytoremediation in heavy metal-contaminated soils focusing on the importance of "mobile/bioavailable" fractions of heavy metals in the soil. Despite the numerous articles appearing in scientific journals, very few field applications of phytoextraction have been successfully realized until now. To overcome the imbalance between the technology's potential and its drawbacks, there is growing interest in the use of plants to reduce only the fraction that is the most hazardous to the environment and human health, which is to target the bioavailable fractions of metals in soil.

At a first glance phytoextraction and phytostabilization seem to have a different goal and, regarding many practical aspects, they indeed do. But despite this, it can be stated that both approaches aim at reducing the amount of mobile/bioavailable heavy metal fractions in the soil. In phytoextraction this is done by removing such fractions and in phytostabilization this is done by reducing heavy metal mobility and bioavailability without removing heavy metals. In the case of phytoextraction, the action of plants only targets the mobile/bioavailable fraction unless other "stronger actions" are taken, e.g., the use of additives to increase the heavy metal mobility, making them more available for plant uptake. Plants can, using their absorbing roots, deal only with the "plant-available" fractions, which can themselves be manipulated by chemical additives or biological action. Moreover, such fractions can strongly vary among different plant species and even varieties. Some plants used in phytoextraction, so-called hyperaccumulators, apparently have the

capacity to modify the mobility/bioavailability of heavy metals in the rhizosphere and seem to have access to basically non-plant-available heavy metal pools in the soil as well. "*Bioavailable Contaminant Stripping*" (BCS) firstly discussed by Hamon and McLaughlin (2003) can be further developed as a remediation approach which is focusing at the removal of all actually and potentially bioavailable heavy metal fractions (see Chap. 13).

Monitoring the mobility and bioavailability of inorganic pollutants (including heavy metals) in contaminated soil provides important information regarding the fate of these contaminants in the environment, time-dependent changes in heavy metal speciation, mobility towards the water table, and ecotoxicological risks (Environmental Agency 2004; Mulligan and Yong 2004). Some authors tend to promote that risk assessment of soils should consider both mobile and bioavailable fractions of heavy metals, which of course depends on the definition of bioavailability (Wahle and Kordel 1997). Despite such considerations, it remains clear that total concentrations of heavy metals in soils are poor indicators of heavy metal toxicity since heavy metals exist in different solid-phase forms that vary considerably in terms of (potential) bioavailability (Nolan et al. 2003). Phytoextraction has proved to be effective, relatively straightforward, and inexpensive compared to other procedures for extracting bioavailable metal fractions from soils. Bioavailable heavy metal fractions, removed by plants, probably correspond to fractions of soil heavy metals that are most prone to affect the soil ecosystem. However, there are surprisingly few reports which show that bioavailable fractions of heavy metals in soils are indeed reduced after concluding a phytoextraction project in the field (Bañuelos et al. 2011; Willschera et al. 2012). As other bioavailable heavy metal fractions can be slowly released by nonmobile heavy metal fractions in the soil (aging), a longer term decrease of the bioavailable fraction might be difficult to observe experimentally. Moreover, this is an argument for considering both mobile and immobile (bioavailable and potentially bioavailable) heavy metal fractions in the soil, when estimating risks.

More data are available regarding phytostabilization; Phytostabilization often uses chemical additives to immobilize heavy metal mobile fractions, especially at heavily polluted sites, which are initially without vegetation due to heavy metal phytotoxicity. Such immobilization is a prerequisite for plant growth. Immobilization therefore has to be measured and monitored.

2.2 How Can We Use Phytoextraction?

2.2.1 Technology Description

Phytoextraction refers to the translocation of metal contaminants from soil up to the above-ground tissues by the root system. After plants have grown for a certain period, they are harvested and may be incinerated to recycle the metals.

This procedure, repeated several times, brings soil contaminant levels down to below legally acceptable limits (Chaney et al. 1997). The time required for remediation depends on the type and extent of heavy contamination, the duration of the growing season, the amount and characteristics of the produced biomass, and the plants natural capability for heavy metal accumulation. Two different strategies can be used (Lombi et al. 2001; Robinson et al. 2003a): continuous phytoextraction—using natural metal hyperaccumulator plants which absorb, translocate, and accumulate an enormous amount of metals during their entire life period without visible toxicity symptoms (Baker and Brooks 1989; Brooks 1998); assisted phytoextraction—the accumulation process is induced in tolerant plants by the increased contaminant bioavailability in soil (Blaylock et al. 1997). Synthetic amendments such as chelates (e.g., EDTA, EDDS, NTA—Cooper et al. 1999; Evangelou et al. 2007), organic acids (e.g., citric acid), or ion competitors (e.g., phosphate—Tassi et al. 2004) added to the soil enhance metal bioavailability, although the soil microbial community is usually neglected and there is a potential risk of leaching of metals to groundwater (Dickinson et al. 2009; Evangelou et al. 2007).

Generally, phytoextraction is only applicable to sites containing low-to-moderate levels of metal contamination. Effective phytoextraction requires both plant genetic ability and optimal soil and crop management practices (Di Gregorio et al. 2006; Tassi et al. 2008; Pedron et al. 2009). *Thlaspi caerulescens* (Cd and Zn hyperaccumulator) and *Brassica juncea* (heavy metal accumulator) are examples of species that well represent the two phytoextraction strategies described above. Metals such as Ni, Zn, Cu, and As are the best candidates for removal by phytoextraction, although Cd, Pb, etc., have been extensively studied as well. Genetic engineering studies have been performed to manipulate plant accumulation with the overexpression or knockdown of membrane transporter proteins (Rogers et al. 2000).

The accumulation of hazardous plant biomass must be disposed of, in order to minimize environmental risk. The waste volume can be reduced by thermal, microbial, physical, or chemical means such as composting, compaction, or thermo-chemical conversion processes (combustion, gasification and pyrolysis). Recycling the biomass from phytoextraction for fuel and other uses cuts down on the need for landfills and provides the contaminated site with an economical value. Added value to the phytoextraction process could be obtained by combining the biomass produced as an energy source, resulting in an ore after incinerating the residual biomass. This would be possible in the case of phytomining, a particular example of phytoextraction. Phytomining involves the exploitation of subeconomic ore bodies using hyperaccumulating plants. For instance, the species *Alyssum bertolonii, Berkheya coddii* have a high potential in extracting Ni because of their high biomass and a Ni concentration of 1 % in the dry matter (Robinson et al. 2003b). Other metals such as gold, thallium, and cobalt have been exploited from tailings or other residues of low commercial value (LaCoste et al. 2001; Keeling et al. 2003). Heavy metal phytoextraction refers to the use of plants that can remove contaminants from soil and accumulate them in a harvestable part in a process alongside water and nutrient absorption by roots. Therefore plant biomass

production and the metal concentration in the biomass are fundamental success factors for the practical efficiency of phytoextraction (McGrath and Zhao 2003; Robinson et al. 2003b).

2.2.2 Protocols for Enhancing Metal Phytoextraction

Several strategies for achieving more efficient heavy metal removal have been recently developed such as the enhancing concentration of soluble heavy metals in the soil with the application of synthetic chelate agents (e.g., EDTA). This then leads to an increase in the metal uptake of high biomass crop plants (e.g., *Brassica juncea, Helianthus annuus, Zea mays,* and *Nicotiana tabacum*) (Meers et al. 2005; Di Gregorio et al. 2006; Pedron et al. 2009).

An alternative strategy, to increase the efficiency of the assisted phytoextraction, is to use plant growth regulators (PGRs) to counteract the negative effects of heavy metal stress in growing plants and boost the shoot biomass (Ouzounidou and Ilias 2005; Lopez et al. 2007; Barbafieri and Tassi 2010; Zhao et al. 2011; Barbafieri et al. 2012). PGRs play a major role in cell division and cell differentiation. They can stimulate shoot initiation, bud formation, the growth of lateral buds, leaf expansion, and chlorophyll synthesis. They can also delay leaf senescence, enhance resistance to salinity, low temperature and drought, and induce stomatal opening in some species (Letham et al. 1978; Barciszewski et al. 2000; Pospisilova et al. 2000). The combined effects of EDTA and cytokine resulted in an increase in the Pb and Zn phytoextraction efficiency (up to 890 % and 330 %, respectively, compared to untreated plants) and up to a 50 % increase in foliar transpiration (Tassi et al. 2008). Cytokinins have also showed potential use for the increasing of Ni phytoextraction capability in *Alyssum murale*, a well-known Ni hyperaccumulator (Cassina et al. 2011). Application of exogenous PGRs was examined as a viable technique to increase the efficiency of plant metal extraction from contaminated soils. However, further experiments are needed to increase the knowledge of the dynamics of the transport mechanism involving metal uptake, since this mechanism is dependent on plant characteristics and environmental parameters. In order to increase the efficiency of phytoextraction, fertilizers can be used to enhance the productivity of selected plants; positive results have been reported recently in the case of the boron-contaminated soils (Giansoldati et al. 2012).

2.2.3 Experimental Protocols for Phytoextraction: Applicability Test at Different Scales

In practice there are always many variables that render each situation "site-specific," so cases in which it is possible to skip feasibility test and proceed to large scale field projects are very rare. In general, the following sequential test steps are applied:

Table 2.1 Micro steps characterizing each phase in a phytotechnology

Sequential period	Type of investigation
Ante operam phase	Site characterization
	Plant and treatment selection
	Organization and preparation of site intervention
	Sowing
	Control of plant growth
In itinere phase	Agronomic care and administration of any fertilizer
	Administration of the chelating agent if necessary
	System monitoring
	Plants harvesting
Post operam phase	Safety of the site
	Waste management
	System monitoring

- *Ante operam* phase (preoperational)
- *In itinere* phase (during the process)
- *Post operam* phase (post-operational)

Each of these phases is characterized by micro-steps aimed at providing the necessary basic information for site characterization. Table 2.1 shows the micro-steps characterizing each of the three main sequential steps, above. In Fig. 2.1, is shown the flow chart of the procedure for the evaluation of the applicability of in situ phytoextraction. The efficiency of phytoextraction is difficult to assess and depends on the nature of contaminants, additive specifications (if used), plant characteristics, and the environmental and soil conditions. To better enhance phytoextraction efficiency, preliminary tests at a laboratory scale and at a greenhouse scale are fundamental, but treatment, biomass, and plant performance are also severely influenced by local environmental conditions. For these reasons, field tests for phytoremediation applicability should be planned for a more realistic estimation of its effectiveness at a specific contaminated site. As for other technologies, treatability could require time and money, but results are fundamental and can be responsible for the success or failure of the project, and can at the end reduce costs. A scheme that could be adopted is subdivided in three steps, which is shown in Fig. 2.2 and briefly indicated below:

First step: characterization of chemical and physical characteristics of the soil matrix
Second step: selection of plant species and/or treatments to be used in phytoextraction
Third step: evaluation through a field-scale pilot test

The first step should be conducted directly on the specific contaminated site in order to evaluate the level of contamination, the agronomic characteristics, and a screening of the indigenous vegetation. The following analyses have to be carried out:

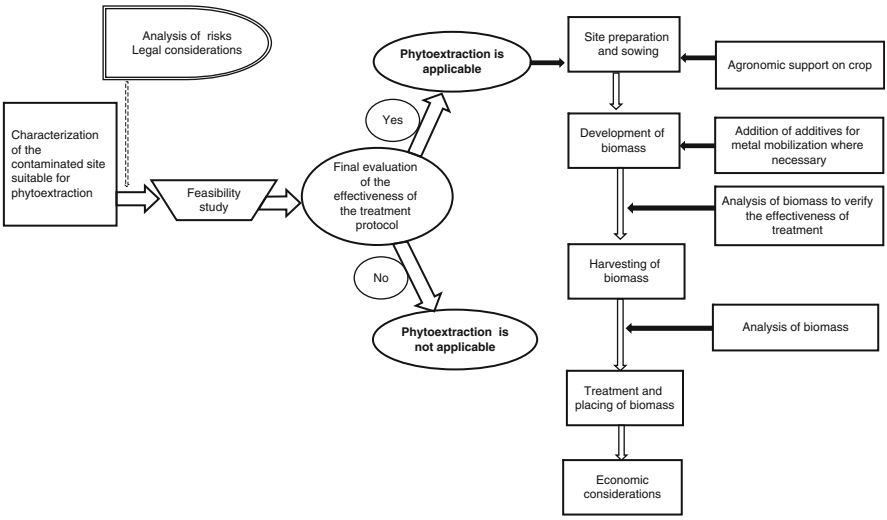

Fig. 2.1 Diagram of applicability of the phytoextraction procedure in situ

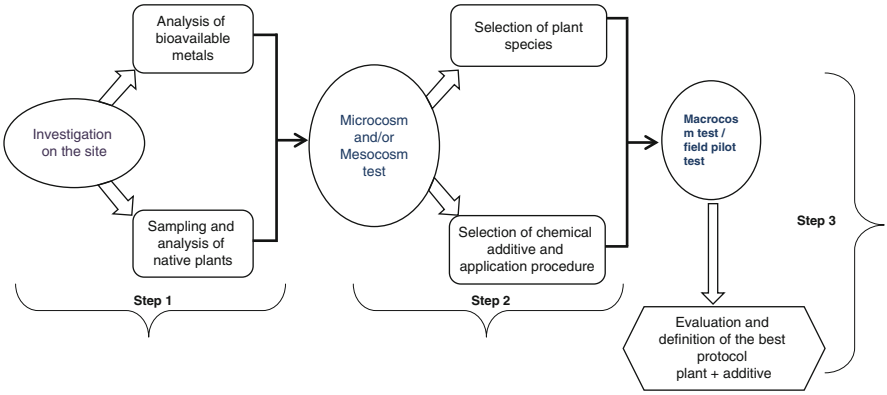

Fig. 2.2 Diagram of the "feasibility study" of phytoremediation technique for soils/sediments contaminated by metals

- Determination of soil biogeochemical parameters together with the agronomic characteristics to verify the status of the soil matrix and to evaluate the potential for plant growth.
- Evaluation of the mobility/bioavailability of contaminants in relation to plant action.
- Determination of contaminants contents in the indigenous plants.

After the first and preliminary evaluation, the treatability test needs to pass additional tests (see Fig. 2.3) for the selection of the best protocols to adopt.

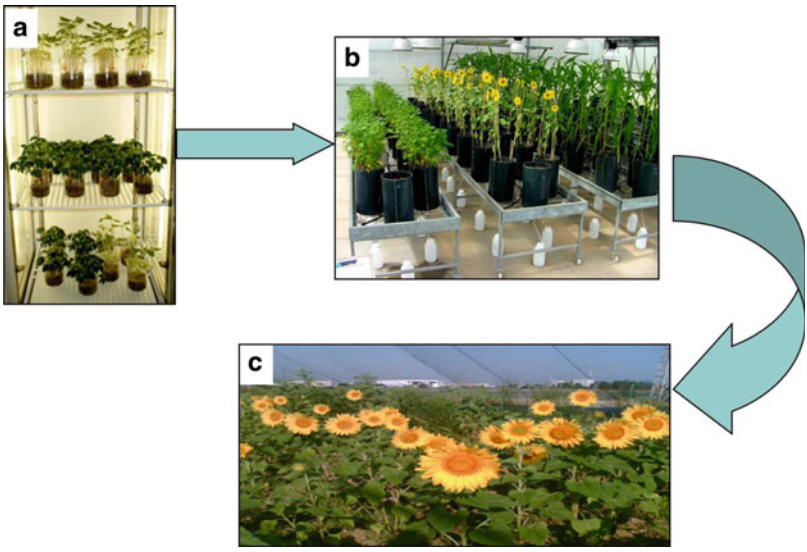

Fig. 2.3 Site-specific feasibility test: (**a**) microcosm, (**b**) mesocosm, (**c**) field test

- Microscale/Lab test (microcosm, Fig. 2.3a): at first the screening test to select the most suitable plants and treatments and to verify eventually heavy toxic effects of the contaminated matrices.
- Mesoscale/Greenhouse (mesocosm, Fig. 2.3b): the most effective protocols (plant plus treatment) tested at the microscale are further investigated at a more "realistic scale" as plants can grow to the end of their vegetative cycle in bigger pots under controlled conditions (in a greenhouse). It allows for the verification of the efficiency of a complete plant and moreover (as pots are provided with leachate collectors) it is possible to check the mobility of contaminants in soil core profile.
- Macroscale/pilot trials (lysimeter, field test, Fig. 2.3c): have to be carried out to verify the performance of the protocols (plants plus treatments) selected from the best performances observed during the mesoscale tests. This last stage allows for the monitoring of plant growth biomass production and contaminants uptake verifying how the local and specific site conditions can influence the phytoremediation process. Moreover appropriate measures can be selected for biomass treatment protection of the area. Uncertainty in the process should be taken into account due to the uncontrolled weather conditions that cause diverse plant response to stress (Tassi et al. 2011; Barbafieri et al. 2010; Barbafieri and Raffaelli 2010).

During all test phases it is very important to monitor reduction of contaminants from the soil as this is main critical success factor of the whole process. This determination if often "forgotten" in scientific articles albeit that it reflects the real effectiveness of the applied phytoextraction protocol. Few show the metal

reduction in soil after a phytoremediation treatment. Pot experiments by Ye et al. 2011 showed a reduction of about 11–38 % after 9-month period of *Pteris vittata* growing for arsenic potentially available (phosphate extractable) and 18–77 % in soil pore water As. Tassi et al. 2011 reported a reduction of 45 % of bioavailable boron after two consecutive growing cycles in microcosm test. Cassina et al. 2012 reported a reduction of 33–45 % of mobile mercury after one growing of *H. annuus* and *B. juncea* respectively in microcosm pot simultaneously treated by cytokinin and thiosulphate. In field experiments this approach is often not considered. The main cause is the high heterogeneity of metal distribution in contaminated soil. Blaylock and Elless 2009 reported a 5-year field study on arsenic removal. But after different sampling grid conducted after each growing season to verify the arsenic removal from soil, they do not observe a significant arsenic removal due to the high soil heterogeneity in arsenic content. The sampling variability challenges the phytoremediation evaluation when approaching a study of mass balance in field experiments (Brus et al. 2009; Van Nevel et al. 2007).

2.2.4 Decision Support Systems

For phytoextraction, a very important critical success factor is the duration of a phytoextraction, i.e., the period between starting the process and the moment when the total concentration or the bioavailable concentration of heavy metal(s) has reached regulatory target levels for soils (Koopmas et al. 2007). To use the total or the bioavailable concentration as target value depends on the legislator's demands; total or bioavailable fractions are determined by standard extraction procedures, e.g., a diluted calcium chloride extraction to mimic plant-availability (Römkens et al. 2009). Many authors simply use a linear phytoextraction model in which the amount of phytoextracted heavy metal is assumed to be independent of the actual heavy metal concentration in soil or soil solution at a certain stage during phytoextraction. Such an approach is definitely a gross simplification which in most cases will underestimate the real phytoextraction duration. It is more probable that the phytoextraction rate in the case of non-hyperaccumulators depends on the actual supply of plant-available heavy metals in the soil, which steadily decreases during the phytoextraction duration. In the case of hyperaccumulators the story might be different; as uptake by such plant species is assumed to be (not only) supply-driven, as "active" processes in the plant root zone may play a role as well. Anyway it is not very likely that a simple model can easily predict phytoextraction duration for both types of plants. Instead of this an experimental protocol can be used, based on mixing the polluted soil with different amounts of clean soil with the same general composition and determine after a period of aging both the plant-available heavy metal concentration (by chemical extraction) and the actual uptake by the chosen phytoextraction plant species. Albeit time-consuming (several months), it results in a better prediction of phytoextraction duration than just using a linear model. Results of such tests also confirm the hypothesis that a nonlinear model is more

likely to predict phytoextraction duration and, more important that thus predicted durations are 20–50 % longer than when the linear model is used. It should be noted that slower processes releasing "new" plant-available fractions from the soil matrix cannot be predicted by this procedure. It may be obvious that phytoextraction duration is an important indicator and decision instrument for phytoextraction, but it is just as obvious that costs play an important role as well (Koopmas et al. 2007; Koopmans et al. 2008a).

2.3 How Can We Use Phytostabilization?

2.3.1 Technology Description

Phytostabilization aims at the use of plants to reduce the impact of soil pollutants on adjacent environmental compartments, including water bodies, agricultural land, etc. Phytostabilization is most effective on land which is highly contaminated by heavy metals, other (in)organic pollutants, and also crude oil residues. Such land is characterized by marginal or nonexistent vegetation and by degenerated soil and surface ecosystems; such land therefore is highly prone to serve as a secondary pollution source due to high wind and water erosion rates and high levels of surface run-off and leaching to the groundwater (Berti and Cunningham 2000; Barbafieri et al. 2011). Phytostabilization of such land areas can be defined as a set of measures which permit re-establishment of vegetation and which at least include the use of chemical/biological soil additives and introduction of productive plants or natural vegetation. In its simplest form, it consists of the addition of adsorbing materials and/or other chemicals which reduce the plant-available fraction of heavy metals and therefore reduce phytotoxicity; the natural vegetation can then return with or without human assistance. An example is the re-establishment of a natural perennial vegetation cover on extremely polluted soil in Poland (up to 4 % of heavy metals) after just adding substantial amounts of rock phosphate and lignite to the soil (Kucharski et al. 2005); see Fig. 2.4. The benefits of such a vegetation cover are obvious. Wind erosion rates are decreased and heavy metals are no longer transported to residential areas and gardens nearby the site.

Leaching is decreased by reducing vertical water transport in the soil as a result of phytoevaporation in combination with a lower mobility of heavy metals after addition of adsorbents. The main risk of the re-establishment of such natural vegetation covers on extremely polluted soil is high uptake of heavy metals by the (hyperaccumulating) plants which can survive on the site and subsequent food-chain contamination. At this specific site in Poland, the non-hyperaccumulating perennial grass gradually won the competition with a hyperaccumulating non-perennial weed, so that food-chain contamination was not a problem anymore after some time. The main disadvantage of phytostabilization from a legislator's point-of-view is the fact that the pollutant is not removed from the soil, but only

Fig. 2.4 Heavily polluted site in Poland (4 % heavy metals) before (*left*) and 1 year after (*right*) application of lignite and phosphate rock. Perennial grass and flowering hyperaccumulating species start growing spontaneously

turned less harmful. The main problem legislators have with this is the fact that in the future, heavy metals may become mobile again and once again can cause environmental problems. Another disadvantage is the fact that the land will remain unproductive on the longer term which gives a longer term management burden to authorities or problem-owners. The reason why phytostabilization remains a good polluted land management option despite the above-mentioned disadvantages is the fact that other options are absolutely not possible due to high costs.

A method to reduce or to completely mitigate the longer term polluted land management costs is to grow non-food crops on the polluted land; this includes energy crops and especially energy crops which provide a perennial vegetation cover (grasses, woody species) and do not cause food-chain contamination problems. Recent research in China and Vietnam has shown that growing energy crops on polluted land can be made profitable (considering the low economic value of the land) even if crop yields are lower than on good agricultural soil. Figure 2.5 shows an energy crop test site in China, nearby a copper/zinc smelter. An interesting example of the interrelation of productive crops and natural vegetation is the effort by Chinese researchers to grow energy crops on extremely polluted (copper, arsenic) mine tailings in Tongling (Anhui, China). After adding rock phosphate and liming the tailings, different tested potential energy crops grew but provided only very low yields which made the whole process economically nonviable. However, after dismantling the energy crop test area, abundant natural vegetation recovered on the site, which has been bare during decades. So no economic profits could be obtained, but the natural vegetation cover which started to reappear did not require high management costs and at the same time reduced the transport of pollutants to neighboring paddy field by decreasing erosion rates and controlling leaching.

2.3.2 Protocols for Phytostabilization

The principal critical success factors for the phytostabilization process are:

1. The effectiveness of the soil additives regarding their effect on reducing the mobility/bioavailability of heavy metals in the soil at the polluted site.

Fig. 2.5 Energy crop demonstration site in the vicinity of make-shift copper/zinc smelters in Fuyang valley (Zhejiang, China)

2. The capacity of the proposed crops or local natural vegetation species to grow on the polluted soil after application of the additives, mainly concerning phytotoxicity.
3. The price of the used additives in combination with the duration of their effectiveness; generally unpolluted waste materials like compost, fly ash, etc., are considered the best option.
4. The longer term effectiveness of the proposed additives and the need to be effective for a longer period (it is possible that the system on the longer term does not need the additives any more).
5. The risk of food-chain contamination induced by the selected plant species.
6. The capacity of the selected plant species regarding their erosion mitigating potential, with special emphasis on all-year effectiveness (perennial vs. non-perennial).
7. The need of fertilizers and pesticides to sustain healthy growth of the selected plant species.

The last five issues (3–7) are general characteristics of additives/plant species and therefore can normally be assessed adequately on the basis of a literature check and/or a very simple decision support system containing literature dataor simply based on an expert opinion, which offers the advantage of integrating the different issues.

Factors 1 and 2, however, are highly site specific and do need preliminary laboratory tests. Such laboratory tests can be a simple series of solvent extractions of the soil/additive mixture (with and/or without aging of the mixtures) especially to chemically assess heavy metal mobility and plant-availability. A simple test to assess potential phytotoxicity is the standard barley root elongation test (see

Fig. 2.6 Barley root elongation test. Phytotoxicity increases from left to right dependent on the used additive mixture

Fig. 2.6) (Kapustka 1997). The root development of barley seeds is highly sensitive to stress caused by pollution and the root length is a good indicator of such stress. The picture shows such a standard test. Using this cheap, fast, and technically easy test many soil/additive combinations can be assessed in a relatively short time and the most suitable combinations can be selected, also taking into account the other success factors, especially the price and local commercial availability of the additives. After this, the best performing additives with the optimum application rates can be tested in pot experiments or small lysimeter studies using the proposed vegetation types (natural species or production crops) to assess crop performance. Accumulation of heavy metals in the crops (issue v) can then be assessed easily as well. When performing these preliminary tests, a check of site heterogeneity has to be carried out as well. If the site is very heterogeneous regarding soil biogeochemical characteristics and pollution levels, it can be decided whether it is (economically) most viable to investigate and apply different phytostabilization schemes to account for the spatial differences in site characteristics or to physically homogenize the upper soil layer, possibly in combination with additive application.

2.4 Conclusions and Recommendations

- In most cases, phytoextraction still requires a long time to attain target pollutant levels in the soil which satisfy the legislators. Therefore commercial applications are being hindered not only by a lack of legal acceptance of the technology as a soil remediation option but also because of the often unpredictable financial burden over a long period of time. These constraints can only be overcome if it can be shown to policy makers that the risk to the environment at

the end can be effectively eliminated. To add a standard ecotoxicology test as a monitoring tool as an integrated part of a phytoextraction project may help to lead to technology acceptance. Research reports and reports on feasibility studies should not only focus on plant accumulation and translocation data but also on an effective reduction of different heavy metal fractions in the soil; they should also provide sound heavy metal mass balances to show that no leaching and other losses occurred.

- Decision Support Tools are not commonly used to help decision-making on which approach is the most appropriate for a specific polluted site. Some tools mainly focus on "hard technology" (e.g., DARTS developed by ICS-UNIDO) and do not specifically deal with phytotechnologies. To improve the use of such a specific Decision Support Tool to decide upon the best approach when phytotechnologies already have been selected for remediation, has to be further developed, to include a database for calibration and validation based on real experimental phytoremediation field trials.
- An agreement on a regulatory base for the use of remediation techniques which only reduce the concentration of heavy metal fraction which pose the major human health and ecosystem risks still has to be developed in many countries. The scientific community already agrees upon the need to do so. Such a regulatory basis will greatly facilitate the introduction of phytotechnologies as an accepted method to reduce risks caused by heavy metals in soils. It will also avoid that phytotechnologies are used where and when they are not appropriate and, on the contrary, avoid situations where more invasive and expensive technologies are used where phytotechnologies represent a better option. Major hurdles for the successful use of remediation approaches based on reduction of bioavailable heavy metal fractions in the soil, which include phytotechnologies, continue to be mainly political and regulatory rather than scientific.
- Regarding phytostabilization, the need of a regulatory framework is even more pressing than in the case of phytoextraction. The reason is that in phytoextraction the bioavailable heavy metal fraction in the soil is effectively removed, which satisfies regulators and public opinion. This is not the case with phytostabilization. Introducing phytostabilization on a broader scale should focus on the following issues:
  - Stressing the need of doing something to stop/reduce the transport of heavy metals from extending extremely polluted sites to cleaner adjacent environmental compartments. Emphasizing that hard technological clean-up is no option, due to extreme costs and emphasizing that dig and dump is not a sustainable solution.
  - Putting emphasis on the fact that there are no other options (except capping in combination with clean-up of groundwater) than phytostabilization and revegetation to improve the situation of extremely polluted extended sites.
  - Promoting the possibility of making phytostabilization economically sustainable on the longer run by using perennial non-food crops like deep-rooting

high biomass production grasses (Miscanthus, Vetiver) and tree species to be used for energy production. Stress the added value of the combination of economic sustainability and erosion control.

- The development and application of phytotechnologies as an environmentally sound approach involves a number of additional challenges. These include the development of local capacity to understand and apply phytoremediation technologies and make them suitable for local economic and environmental conditions and the establishment of an effective regulatory framework. In some countries, there is a lack of experience in the use of phytoremediation. This is often coupled with a lack of data, performance standards, and cost–benefit analyses regarding phytotechnologies. In summary, there is a need for:
  - Appropriate phytoremediation technologies and techniques applicable to different geographic regions with varying weather conditions
  - Site characterization, clean-up, and technology selection criteria, including decision support tools
  - Assessment and evaluation methods that can be applied to determine the applicability and appropriateness of various phytoremediation techniques
  - Local training for environmental remediation practitioners on the planning and implementation of phytoremediation schemes.
- Extended complex polluted sites, including mining sites and smelter areas, often are characterized by a high spatial variation in pollutant levels and soil parameters, relevant for determining mobility, and bioavailability of heavy metals. Developing such sites gives good chances for phytotechnologies to be among a mix of invasive and noninvasive techniques and approaches to be used for site development, especially when creating parks and recreational areas.

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Chapter 3
Metal/Metalloid Phytoremediation: Ideas and Future

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Heavy metals and metalloids constitute a serious ecological concern in almost any environmental matrix (Fu and Wang 2011). The actual problem connected with trace elements results from the fact that they are readily transported to living organisms and have an adverse effect on their physiology. Taking into consideration periodical economic recessions and permanent demand for energy, it is necessary to develop modern methods based on biological, neutral and relatively cheap measures, characterised by high efficiency (Witters et al. 2012). Biological methods, including phytoremediation, are often considered as unconventional in relation to other methods (physical, chemical and mechanical), but they have a considerable potential, which even at the commonly acknowledged limitations in their applicability, ensures their dynamic development (Singh and Prasad 2011). The scope of issues investigated by authors of research papers concerning phytoremediation is extensive and in the last 20 years has been considerably modified, aiming at ensuring high process efficiency within a relatively short time, as well as simplicity of the adopted solutions and low costs (Glick 2010). As with any method, phytoremediation has its own limitations connected with, e.g. maintaining relatively high effectiveness over long time periods. This fact is obvious, particularly when comparing this approach with technical or semi-technical methods, exhibiting high effectiveness and rapid rate of operation (Peng et al. 2009). However, biological methods will develop even faster than what we observe at present, as is evidenced, e.g. by the rapidly growing number of research papers published on the subject in recent years. Our aim in this study to present several essential problems concerning phytoremediation, insight into

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which will provide further directions of development for this reclamation method in case of soils contaminated with heavy metals. A crucial element presented in this paper and frequently used by opponents of biological methods is related to the residue after the phytoremediation process.

3.1 Phytoremediation: Yesterday, Today . . . Tomorrow

In recent years we have observed an increased interest in hyperaccumulators, although despite knowledge gained on molecular/cellular uptake mechanisms of selected trace elements (Jabeen et al. 2009; Memon and Schröder 2009), their translocation to individual aboveground organs and detoxification, we still need to deal with the problem of very limited biomass of these plants. Initial studies concerned herbaceous plants, but due to their low biomass, significantly contributing to increased costs of practical application of these plants, interest was quickly shifted to include also woody plants (Yadav et al. 2010). Other aspects of enhancing the phytoremediation potential are connected, e.g. with modification of contaminated substrate to facilitate sorption of metals/metalloids from soil by the plant root system (Wang et al. 2009; Zhao et al. 2011; Mleczek et al. 2012), the application of microorganisms (Weyens et al. 2010) and short rotation coppice (SRC) as fast growing tree species with a significant biomass increase (Dimitriou and Rosenqvist 2011). This latter aspect seems to be of particular interest, as it is connected with the increased demand for energy from renewable sources, crucial particularly in recent years. Selected plant taxa from *Populus* or *Salix* species are characterised by a significant increment in biomass, especially in areas with high ground water levels, and at the same time relatively high capacity to absorb heavy metals/metalloids (Adegbidi et al. 2001).

Renewable energy sources (RES) are playing an increasingly important role in the generation of primary energy in the European Union. In the years 2001–2009, generation of energy from renewable sources increased from 10.6 % to 18.3 %. Biomass became the main source of renewable energy. In this respect one of the most important EU documents was the Directive of the European Parliament and Council no. 2009/28/EC of 23 April 2009 (the $3 \times 20 + 10$ climate and energy package). New objectives were specified in this package concerning the use of renewable energy and greenhouse gas emissions. It was assumed that by the year 2020 the share of renewable energy would increase to 20 % (a significant increase in the use of non-forest biomass in energy generation) in the total balance of energy consumption in the EU. In such a case biomass from phytoremediation—with the application of additional measures limiting further heavy metal transport to the environment—may significantly increase the amount of biomass required to meet the stipulations of the directive.

In recent years, studies on phytoremediation have focused on the use of bacteria and mycorrhizal fungi as well as genetic modifications described in

one of the points below (Kotrba et al. 2009; Miransari 2011; Prasad et al. 2011). Selected bacterial strains, i.e. plant growth-promoting rhizobacteria (PGPR) such as *Azospirillum*, *Rhizobium*, *Enterobacter* or *Arthrobacter*, may be used to increase plant growth (Farina et al. 2012). These organisms are capable of cooperating with plants by reducing the adverse effects of toxic substances on their growth, stimulation of nutrient transport required for appropriate plant growth or formation of such compounds (Jha et al. 2011; Tang et al. 2012). The presence of PGPR in the rhizosphere of plants used in phytoremediation is particularly essential, as they have a positive effect on the development of the root system (stimulation of growth and thus also uptake of nutrients from soil) and limiting plant ageing processes by ethylene inhibition, i.e. blocking of its production by ACC-deaminase activity from bacteria (ACC-1-aminocyclopropane-1-carboxylate) (Saleem et al. 2007). PGPR are capable of producing many phytohormones, e.g. gibberellins, cytokinins or indole-3-acetic acid (IAA) (Ma et al. 2011). For this reason, it may be assumed that these organisms in the near future will be important subjects of studies on enhancing resistance of plants growing in areas polluted with metals/metalloids, as well as maintaining or increasing their growth (preventing a reduction of biomass under conditions adverse for plant growth).

Another interesting group of growth promoting organisms, at the same time enhancing efficiency of heavy metal uptake from polluted areas, comprises endophytic bacteria (gram-positive and gram-negative) as well as siderophore-producing bacteria (*Pseudomonas putida, Bacillus megaterium* or *Ralstonia metallidurans*). The former are organisms colonising plant tissues having a positive effect on plant growth and enhancing tolerance to the presence of toxic trace elements. They exhibit several significant traits, e.g. they promote the uptake of nutrients required for appropriate plant growth and they have a positive effect on the capacity to limit the adverse effect of pathogens (Rajkumar et al. 2010). An even more interesting aspect of recently undertaken research is connected with the applicability of low molecular chelators produced by fungi, bacteria and plants, exhibiting high affinity to selected metal ions (Al, Cd, Cu, Fe or Zn). Siderophore-producing bacteria (SPB) are also capable of stimulating plant growth, yielding an increase of biomass and enhanced resistance to the presence of heavy metals. They exhibit a capacity to increase the amount of metals absorbed by plant tissues or enhance plant tolerance by stimulating growth of individual plant organs (Rajkumar et al. 2010). In the near future, numerous studies on phytoremediation are likely to focus on the application of new specialised organisms which will promote plant growth and development and at the same time will protect plants against the adverse effect of heavy metals present in the soil. Moreover, such studies conducted in situ will make it possible to develop optimal guidelines for the application of plants selected for growing in polluted areas in order to achieve the highest possible efficiency of heavy metal uptake from soil.

Table 3.1 Salicylic acid (free and glucosidal) contents in leaves of *Salix viminalis* L. cv. "Cannabina" cultivated hydroponically in Knop's medium containing Cu^{2+} and Ni^{2+} soluble salts (Drzewiecka et al. 2012; Gąsecka et al. 2012)

	Metal concentration in medium [mM]			
	0	1	2	3
	Free SA			
Cu	0.33 ± 0.01	1.16 ± 0.07	4.57 ± 0.09	7.94 ± 0.06
Ni	0.36 ± 0.03	12.23 ± 0.11	5.72 ± 0.08	16.89 ± 0.04
Cu+Ni	0.92 ± 0.15	2.69 ± 0.29	6.35 ± 0.13	10.73 ± 0.47
	Sum of free and glucosidal SA			
Cu	2.23 ± 0.07	4.33 ± 0.32	7.94 ± 0.10	21.07 ± 0.38
Ni	2.44 ± 0.05	38.26 ± 0.11	21.62 ± 0.18	61.31 ± 0.31
Cu+Ni	2.59 ± 0.05	10.41 ± 0.12	7.78 ± 0.79	35.69 ± 0.81

3.2 Impact of Endo- and Exogenous Salicylic Acid on Plant Tolerance to Metallic Ions

Salicylic acid (SA) is one of the phenolic metabolites widely distributed in plants. It possesses a complex function in regular plant growth and development, as well as in tolerance mechanisms against numerous environmental factors of both biotic and abiotic nature. Biotic stressors cause the enhanced biosynthesis of salicylic acid to develop the hypersensitive response (HR) (suicidal auto-oxidation at infection site), and further an intra- and interplant systemic acquired resistance (SAR) with the induction of pathogenesis-related proteins (PRs) (Raskin 1992). Salicylic acid function in plant response to abiotic factors directly causing oxidative damage (mainly of anthropogenic origin, e.g. tropospheric ozone or metallic ions), remains the subject of on-going debate. Pál et al. (2005) proved the elevated biosynthesis of salicylic acid upon cadmium stress in young maize (*Zea mays* L.) seedlings. After 7 days of cultivation in medium containing $Cd(NO_3)_2$ (10, 25 and 50 μM), increased concentrations of free and bound benzoic (BA), *o*-coumaric (*o*-HCA) and salicylic acid were observed in leaves, without changes in SA content in roots, where only 50 μM Cd treatment enhanced the accumulation of free *o*-HCA and bound BA. In our studies, the exposure of basket willow (*Salix viminalis* L.) to copper and/or nickel (0.5–3 mM as nitrate salts) in hydroponic solution resulted in a substantial increase of free and glucosidal SA contents in leaves. However, induction of salicylic acid biosynthesis in photosynthetic tissue (and/or upstream transport from roots) differed significantly for both metals analysed. Furthermore, it can be assumed that synergistic and antagonistic interactions between metal toxicity occurs taking into consideration the elevation of SA content by these two metals applied simultaneously (Table 3.1).

Freeman et al. (2005) proved the enhanced accumulation of salicylic acid and its up- and downstream metabolites (phenylalanine, cinnamic acid and salicyloyl-Glc, catechol, respectively) across different species of *Thlaspi* showing Ni/Zn hyperaccumulation. Furthermore, elevation of free SA levels in *Arabidopsis*, both

genetically and by exogenous feeding, enhanced post-translationally the specific activity of Ser acetyltransferase (SAT), leading to elevated glutathione (GSH) biosynthesis and, in consequence, increased resistance to nickel. The authors presume that in *Thlaspi* hyperaccumulators, the GSH-mediated Ni tolerance is signalled by the constitutively elevated levels of salicylic acid, and the increased GSH pool allows *Thlaspi* to resist the Ni-induced oxidative stress (Freeman et al. 2004). Furthermore, according to Pál et al. (2002), salicylic acid potentially blocks the activity of phytochelatin synthase to maintain the efficient GSH level to act as an antioxidant. Relatively numerous studies have been conducted to assess the influence of seed priming with SA or its addition to the cultivation medium on metal uptake and plant resistance parameters. Choudhury and Panda (2004) examined the influence of salicylic acid on cadmium tolerance of *Oryza sativa* L. seedlings. Rice seeds were soaked in salicylic acid solution (100 μM) for 16 h before germination and then treated with $CdCl_2$ at concentration of 0, 10, 100 and 1,000 μM in a hydroponic culture. After 24 h of cultivation, cadmium accumulation in roots was greatly (~50 %) lowered for SA primed seeds at the highest Cd concentration in the cultivation medium. Increasing Cd concentration resulted in the gradual decrease of root length and dry mass. However, seed treatment with salicylic acid reduced the negative effect of cadmium on growth parameters, especially on the dry mass of the roots, which was markedly (as much as twice) higher for SA-treated seedlings at each Cd concentration. Simultaneously, seed treatment with SA depleted the membrane damage in roots resulting from lowered generation of excessive H_2O_2. Thus, the content of malondialdehyde (MDA) from lipid peroxidation was greatly lowered by SA down to ~65 % of SA-untreated seeds (at 1,000 μM Cd) and was accompanied by the reduction of catalase, guaiacol peroxidase and glutathione reductase activity. In a study of Belkadhi et al. (2012), salicylic acid pre-treatment of flax (*Linum usitatissimum* L.) seeds markedly alleviated cadmium toxicity to developed seedlings. After 10 days of cultivation, exogenous SA in concentrations of 250 and 1,000 μM lowered cadmium bioaccumulation factor (BAF) in roots and shoots, as well as translocation factor (TF) to the photosynthetic tissue. Furthermore, the total (mainly shoot) dry weight, shoot-to-root ratio and leaf area significantly increased as an effect of seed priming with salicylic acid. Enhanced non-protein thiol (NP-SH) production was observed in flax roots, and decreased in leaves, suggesting a preventative role of salicylic acid in Cd uptake, sequestration and translocation processes. Popova et al. (2008) investigated the effect of SA pre-treatment on cadmium toxicity to pea plants (*Pisum sativum* L.). Pea seeds were soaked in 500 μM SA for 6 h before germination and then cultivated for 12 days in medium containing $CdCl_2$ at 0, 0.5, 1, 2 and 5 μM. SA treatment significantly lowered cadmium accumulation in pea roots in comparison to SA-untreated seedlings, i.e. from ~480 down to 130 mg kg^{-1} DW at 5 μM Cd, and reduced the inhibitory effect of cadmium on growth parameters (roots and shoots fresh weight). Simultaneously, SA alleviated the negative impact of Cd on photosynthesis and carboxylation reactions and showed a stabilising effect on thermo luminescence characteristics of pea leaves. In addition, at moderate Cd concentrations (1 and 2 μM), SA treatment lowered by about half the metal-induced

biosynthesis of endogenous salicylic acid in pea leaves. Similar results were obtained by Metwally et al. (2003) in the case of Cd-treated barley seedlings (*Hordeum vulgare*). SA-priming treatment (500 μM) of dry caryopses decreased cadmium toxicity and was beneficial for all growth parameters (excluding shoot dry weight), although total Cd in root and leaf tissue remained unaltered. Surprisingly, the addition of salicylic acid to the hydroponic solution for 24 h had a similar positive effect on barley seedlings. In addition, SA reduced the level of MDA in roots of Cd-treated seedlings and increased by 20 % total non-protein thiol content compared to Cd treatment. According to Kováčik et al. (2009), salicylic acid (50 μM) added to the cultivation medium containing cadmium or nickel soluble salts (60 μM) altered the rate of metal uptake and translocation from roots to leaves of chamomile plants (*Matricaria chamomilla*). Cadmium transport to the photosynthetic organs was greatly reduced by SA treatment, but in roots total Cd was found at a comparable level. However, nickel accumulation in chamomile leaves was significantly increased by salicylic acid with simultaneous reduction of its content in roots, indicating distinct modes of salicylic acid action in chamomile response to both metals. Plant treatment with SA altered the activity of phenolic metabolism-related enzymes either in chamomile roots or leaf rosettes. SA enhanced the activity of shikimate dehydrogenase (SKDH) in leaves and cinnamyl alcohol dehydrogenase (CAD) in roots in the case of nickel-treated plants and greatly lowered SKDH activity in roots in the case of cadmium addition. As a consequence, significant changes in composition of chamomile phenolics including benzoic and cinnamic acids were noticed. The accumulation of endogenous SA was strongly induced in plant roots and aerial organs in the case of nickel and salicylic acid simultaneous treatment. In the case of cadmium, exogenous salicylic acid enhanced endogenous SA biosynthesis in roots, but lowered it in leaf rosettes due to the SA-mediated inhibition of cadmium translocation up to leaves.

3.3 Biochemical Responses to Metals

Growth inhibition, water and nutrient imbalance, decrease of photosynthetic activity and oxidative stress are only a few of the multiple effects observed in plants growing in the presence of heavy metals. Metal toxicity is a result of the binding of ions to functional groups in proteins, nucleic acids or lipids, leading to inhibition of their activity or structure disruption, or also from the exchange of essential metal ions from the active centres of enzymes resulting in deficiency effects (Van Assche and Clijsters 1990). In addition, like most stress factors, an excess of heavy metals may lead to the generation of harmful reactive oxygen species which react with macromolecules important for cell functioning (Dat et al. 2000; Clemens 2001; Clemens et al. 2002; Pittman 2005). One of the first effects of metal toxicity is the very well documented increase of reactive oxygen species (ROS) in plants. Normally, ROS produced during different metabolic processes such as photosynthesis are immediately dissolved by antioxidative enzymes and molecules. Increased

amounts of reactive species lead to disruption of cell equilibrium. The level of ROS generation depends on heavy metal characteristic, speciation form, and concentration. Metals can be divided into two groups: redox active (Fe, Cu, Cr, Co) and redox inactive (Cd, Zn, Pb, Ni, Al, etc.) (Hossain et al. 2012). Metals such as Cu or Fe are known to be directly involved in the formation of $O_2^{\bullet -}$ and consequently H_2O_2 and also highly reactive $^{\bullet}OH$ via the Haber-Weiss and Fenton reactions. However, oxidative stress in plants exposed to metals such as Cd or Pb is an effect of their interaction with membrane lipids and proteins, antioxidative enzymes, elements of the electron transport chain and consequently disruption of their functioning (Hall 2002; Metwally et al. 2005; Romero-Puertas et al. 2007). Increased levels of ROS lead to lipid peroxidation, protein oxidation, disturbances in membrane permeability and cell division, but simultaneously they may also function as signalling molecules. Due to its characteristics, H_2O_2 in particular may be perceived as a signalling molecule (Dat et al. 2000). Many authors have observed an increase of H_2O_2 concentration in response to different metals in various plants such as lupine, tomato, *A. thaliana*, barley, pea and bean (Cho and Park 2000; Maksymiec and Krupa 2006; Małecka et al. 2009). The increase of ROS induces activation of antioxidative mechanisms at the molecular and biochemical level or may activate the apoptosis pathway. The activated elements of defence systems differ depending on metals, plant development, organ or tissue. Among the early activated mechanisms, antioxidative enzymes play an important role in maintaining the cell balance. The most studied elements of this system include superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione *S*-transferase (GST; EC 2.5.1.18). In particular, superoxide dismutase, catalase and peroxidase are important to balance the production and elimination of ROS in plant cells. Also participating in the antioxidative response are low molecular weight compounds including ascorbate (AsA) and glutathione (GSH) which can directly quench ROS, cooperate with antioxidative enzymes such as APX, GPX, GST or GR and also regulate gene expression of proteins involved in the stress response.

The level of enzyme activity is dependent on metal concentration and properties, exposure time and tolerance ability of plants. An increase in CAT activity was observed in response to Cd, Fe and Pb in *Nicotiana plumbaginifolia*, *Pisum sativum* and *Brassica juncea* (Minglin et al. 2005; Małecka et al. 2009). On the other hand, Romero-Puertas et al. (2007) reported a decrease of CAT activity in pea plants treated with 50 μM $CdCl_2$. The author suggested that enzyme inhibition was caused by protein oxidation due to metal presence which led to upregulating the transcription of the corresponding gene. Also for other enzymes there is a similar lack of a clear pattern between activity, metal ions and plants. For example, there are reports not only indicating increase of Cu and Zn-SOD activity in tomato, pea and Indian mustard plants treated with Cu, Cd, Pb or Fe (Pich and Scholz 1993; Lin et al. 2007; Małecka et al. 2008) but also describing decrease of enzyme activity in tomato, lupine and pea plants treated with different metals (Romero-Puertas et al. 2007).

Almost all enzymes involved in the removal of ROS are dependent on the presence of certain metal ions in their active centres, e.g. Cu, Zn, Mn or Fe for SOD and Fe for CAT or APX. Excessive levels of free metal ions with similar properties can lead to displacement and ion substitution and consequently inhibition of protein activity. The stunted growth and decrease of biomass observed in plants grown in a polluted environment is the result of numerous changes in cell functioning. A prominent one is disorder in chloroplast structure and functioning. Although most plants accumulate heavy metals in roots and only 1–5 % of absorbed ions are transported to above-ground parts (Piechalak et al. 2002, 2003) even such a small amount has a significant impact on leaf structure and functioning. Chloroplasts in these plants are smaller and the number of both grana and thylakoids is reduced. However, the negative metal effect is not expressed to the same degree in all chloroplasts; particularly exposed are chloroplasts located near the vascular system, where the concentration of metals is the highest (Krupa 1988). There are numerous reports indicating a decline in chlorophyll content during exposure to heavy metals in almond, bean, corn, sunflower, Norway spruce or oak (Nada et al. 2007). This decrease is caused by several factors, for example imbalance in nutrient level or inhibition of enzymes involved in chlorophyll biosynthesis. Due to competition of transporters, disruption of water management and membrane permeability, heavy metals cause disturbances in uptake of elements; it was reported that the most affected is the absorption of N, K, Mg and Mn. The effects on absorption of P, S, Ca, Zn and Fe are more complex; their uptake is related to plant species, environmental stress, pH and soil. For chlorophyll biosynthesis, especially important is decrease in Mg, Fe, Ca and Zn level observed in plants exposed to Cd, Pb, Cu or Mn (Van Assche and Clijsters 1990; Küpper et al. 1996). Presence of cadmium or lead entailed decrease or inhibition of activity of δ-aminolevulinic acid dehydratase and protochlorophyllide reductase, which are involved in chlorophyll biosynthesis (Padmaja et al. 1990; Van Assche and Clijsters 1990). It was shown that heavy metals affected the function of both PSI and PSII, although PSII remains the main target of metal toxicity. The authors reported that the proteins which took protons for photosynthesis in PS II were decomposed and decreased under Cd stress.

Heavy metal stress has been shown to both induce and inhibit expression of various protein genes which results in changes in protein content (Shah and Dubey 1997). Palma et al. (2002) observed a decrease in general protein content during exposure of *Brassica juncea* to high concentrations of Cd and Pb. The author postulated that this may be caused by enhanced protein degradation as a result of increased protease activity, which is found to increase under stress conditions. It is also likely that these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content. Decrease in the protein content has also been found in aquatic plants when treated with metalliferous wastewater (Aravind and Prasad 2005). In plants exposed to heavy metal there was reported an increase of various molecules that can bind with metal ions and form stable complexes, which greatly decreases metal toxicity. It was shown that organic and amino acids (such as citric acid, oxalic acid, succinic acid, aspartic acid, glutamic acid, histidine, and cysteine)

formed soluble complexes with heavy metals and in that form may be transported for example to vacuoles. Metal presence also induces production of specific ligands such as metallothioneins (MTs) and phytochelatins (PCs). A large number of recent studies in plants involving sensitive, tolerant, mutant, transgenic, and hyper-accumulator plants concentrate on glutathione and phytochelatins as key elements in their tolerance and accumulation strategies. Phytochelatins (PCs) were first isolated by Grill et al. (1985) from a cell-suspension culture of *Rauvolfia serpentina*. They have the structure [(γ-Glu–Cys)n–Gly], where n is the number of replications of the (γ-Glu–Cys) units, which is generally in the range 2–11. The enzyme responsible for their synthesis is c-glutamyl cysteine dipeptidyl transpeptidase (phytochelatin synthase: PCS), the substrate of which is glutathione (Grill et al. 1989). The enzyme is also expressed constitutionally but is primarily activated by the presence of heavy metals. First phytochelatins bind metals and form low-molecular-weight (LMW) complexes, then form high-molecular-weight (HMW) complexes with acid-labile sulphur, which are more stable. The HMW metal–PC complexes are then transported to the vacuole, where under acidic pH the metals form complexes with organic acids (citrate, oxalic acid and malate) and probably with amino acids, while the phytochelatins are either decomposed by hydrolases or return to the cytosol (Sanità di Toppi and Gabbrielli 1999). The literature on the relationship between heavy-metal tolerance and phytochelatin synthesis contains many contradictions (Arisi et al. 2000; Pál et al. 2006; Barałkiewicz et al. 2009). Wójcik and Tukiendorf (2011) found that tolerant maize accumulated far less Cd than the more sensitive rice or wheat. The use of more sensitive techniques allowed observation of both oxidised and reduced form of glutathione and phytochelatins in plants treated with cadmium and lead (Barałkiewicz et al. 2009). The authors showed that only Cd was chelated by phytochelatins while Pb bound with proteins and phytochelatins probably play a role as antioxidants. This may suggest that phytochelatins are important components in the detoxification of heavy metals, but they are unlikely to be responsible for metal tolerance, avoidance or hyperaccumulation.

In conclusion, numerous important processes are affected by metal presence and have consequences for plant condition and growth. Some of these mechanisms are specifically involved in defence against heavy metals and their impacts on plant functioning, such as phytochelatins, antioxidants and proline. An important field for further research may be the tolerance mechanism of plants exhibiting hyperaccumulation abilities.

3.4 Genetic Background of Plant Adaptation to and Hyperaccumulation of Metal(loid)s

Two adaptation strategies are observed in plants growing on metalliferous soils: the common “excluder” strategy which restricts accumulation of toxic metal(loid)s to the root, and the more advanced hyperaccumulator strategy of translocation to the shoot. Toxic metal uptake, translocation and accumulation interacts with uptake

and allocation of other nutrients and depends on regulation of genes involved in cation uptake, allocation, sequestration and biosynthesis of metal(loid) ligands. Since the ability to hyperaccumulate metal(loids) (Ni, Zn, Cd, Se, Mn, Co, Cu, Pb, Sb, Tl or As) without toxicity symptoms, shared by about 500 plant taxa growing on metalliferous soils, is of polyphyletic origin, it seems likely that only minor changes in the plant genome can convert it into a hyperaccumulator (Verbruggen et al. 2009). Several species from the *Brassicaceae* family have evolved the ability to accumulate Ni, Zn, Cd, Se and As. Comparative analyses of transcriptome, ionome and metabolome of the model plant *Arabidopsis thaliana* and related hyperaccumulator species is a powerful strategy to investigate adaptive changes in plant genomes leading to metal(loid) tolerance and hyperaccumulation.

3.4.1 Small Ligands

Metal(loid) homeostasis in plants depends on metal binding proteins and peptides, as well as on biosynthesis and partitioning of small ligands such as citrate, acetate, malate, oxalate, phosphate, histidine (His), phytate, glutathione (GSH), phytochelatins and pectates (Krämer et al. 2000). The concentration of histidine and nicotianamine, which form more stable complexes with bivalent cations than organic acids like citrate, is crucial for hyperaccumulation of Ni and Zn (Callahan et al. 2006; Haydon and Cobbett 2007). In *Alyssum lesbiacum* Ni uptake is proportional to His concentration in xylem, and the gene of ATP-phosphoribosyltransferase (ATP-PRT) catalysing the first step in His biosynthesis is constitutively overexpressed, which distinguishes *A. lesbiacum* from its relative non-accumulator *Alyssum montanum*. Overexpression of an *A. lesbiacum* ATP-PRT cDNA in transgenic *A. thaliana* increased Ni tolerance and the pool of free His in the shoot but Ni concentration in neither the xylem sap nor in the shoot was increased, which indicates that additional factors are necessary for Ni hyperaccumulation (Ingle et al. 2005). The high rate of root-to-shoot translocation of Ni in *T. caerulescens* compared to *Thlaspi arvense* seems to depend on enhanced root His concentration and on decreased ability to accumulate Ni–His complexes in root cell vacuoles. Nicotianamine (NA) is a Fe chelator formed from *S*-adenosyl-L-methionine by NA synthase (NAS). The exposure of *Thlaspi caerulescens* to Ni triggers the accumulation of NA in roots. Since neither TcNAS expression nor NAS activity were detected in roots, the NA is most likely translocated from shoots, partially as a stable Ni–NA complex in the xylem sap. Such circulation of NA and Ni–NA chelates cannot be detected in the non-accumulator *Thlaspi arvense*. In *A. thaliana*, NAS transcript levels are upregulated under Fe, Zn and Cu deficiency. In both *A. thaliana* and *A. halleri* (Zn and Cd hyperaccumulator), Zn deficiency induces accumulation of NAS transcript in the shoot (Talke et al. 2006). Under normal growth conditions, *A. halleri* shows high expression of NAS in roots and accumulates more NA. NA is expected to act in the cytoplasm and in the phloem, but in transgenic plants

the enhanced compartmentalization of NA in the vacuole directed the vacuolar accumulation of Zn (Haydon et al. 2012). The increased expression of *A. thaliana* ZIF1 (a vacuolar membrane major facilitator superfamily protein required for basal Zn tolerance) promotes vacuolar sequestration of Zn. In *A. thaliana* ZIF1 overexpressors Zn is immobilised in roots, and the concomitant sequestration of NA impairs the translocation of Fe from leaf vasculature to leaf blade and gives constitutive symptoms of Fe deficiency, similar to biosynthetically NA-deficient plants (Takahashi et al. 2003; Haydon et al. 2012). *A. halleri* and *T. caerulescens* share elevated NAS expression compared to non-accumulators. Suppression of AhNAS2 by RNA interference resulted in reduced root NA accumulation, decrease in root-to-shoot translocation of Zn, increase in Zn-thiol species and reduced accumulation of Cd in leaves (Deinlein et al. 2012). Transgenic rice plants overexpressing OsNAS3 (35S enhancers) accumulate more Fe and Zn in shoots, and two to threefold more Fe, Zn and Cu in seeds, and they exhibit increased tolerance to Fe and Zn deficiencies and tolerance to Zn, Cu and Ni toxicity. OsYSL2 is an iron [Fe(II)]–NA and manganese [Mn(II)]–NA complex transporter, expressed in phloem companion cells and developing seeds, important for Fe translocation, especially in the shoots and endosperm (Ishimaru 2010). Recently Masuda et al. (2012) have tested a combined transgenic approach in rice expressing ferritin from an endosperm-specific promoter, overproducing NA and enhancing the Fe flux through expression of OsYSL2 from the endosperm-specific promoter and sucrose transporter promoter and obtained transgenic plants which exhibited fourfold higher iron accumulation in polished grains (Masuda et al. 2012).

3.4.2 Metal(loid) Uptake, Translocation and Partitioning

A. thaliana genome encodes 15 ZIP transporters. The best characterised, IRT1 (Iron-Regulated Transporter 1), is responsible for root uptake of Fe^{2+} into epidermal cells of the root hair zone (Colangelo and Guerinot 2006). Transcript levels of IRT1 are regulated by local root and shoot-derived long-distance signals. IRT1 transcripts accumulate during the day, indicating the circadian regulation of Fe acquisition (Vert 2003). IRT1, IRT2 and transcripts of other genes involved in Zn and Cd detoxification increase under Fe deficiency (Wu 2012). Transcript levels of plasma membrane IRT3, proposed to transport Zn^{2+} and Fe^{2+}, increase under Zn deficiency and are constitutively overexpressed in roots of the Zn hyperaccumulators *A. halleri* and *T. caerulescens* (Becher et al. 2004; Talke et al. 2006). ZIP1, ZIP2, ZIP3 and ZIP4 mediate Zn uptake in a heterologous system and ZIP4 also Cu.

AtHMA1 and AtHMA6 (members of P1B-type ATPase family) encode a high affinity Cu(I) transporter of the chloroplast envelope (Catty 2011) while soybean AtHMA8 homologue localises to thylakoid membranes (Bernal et al. 2007). AtHMA1 can also be a Zn or Ca transporter (Moreno 2008; Kim 2009). Transcripts

of vacuolar AtHMA3 (similar to prokaryotic Zn^{2+}/Cd^{2+} pumps) are elevated in shoots of *A. halleri* and *T. caerulescens* (Becher et al. 2004; Talke et al. 2006). The hma3 mutant of *A. thaliana* is more sensitive to Zn and Cd, while HMA3 overexpressor plants are more tolerant to Zn and Cd and accumulate more Cd. AtHMA2 and AtHMA4 are localised to plasma membrane pericycle and xylem parenchyma cells (Hanikenne et al. 2008) and participate in loading of Zn and Cd into the xylem for root-to-shoot translocation (Wang et al. 2009). *A. thaliana* hma2 hma4 double mutants are Zn deficient in the shoots. HMA4 is necessary for Zn hyperaccumulation in *A. halleri* shoots (Hanikenne et al. 2008). Transfer of an *A. halleri* HMA4 gene to *A. thaliana* confers Zn translocation into xylem vessels and up regulation of Zn deficiency response genes, but is not sufficient to increase Zn or Cd tolerance (Hanikenne et al. 2008).

The 12 *A. thaliana* MTPs belong to different phylogenetic groups and likely differ in substrate specificity (Delhaize et al. 2007). MTP1 and MTP3 are localised to the vacuolar membrane and probably transport Zn into the vacuole. MTP1 increases Zn concentration in leaves while MTP3 has an opposite effect (Desbrosses-Fonrouge et al. 2005). MTP1 homologues are highly expressed in hyperaccumulator species such as *A. halleri* and *N. caerulescens* (Shahzad 2010). The His-rich cytoplasmic loop of MTP1 may act as a sensor or a buffer of cytoplasmic Zn, and deletion of this loop makes MTP1 hyperactive (Kawachi et al. 2009). Four other *A. thaliana* MTPs are similar to a legume MTP8 of *Stylosanthes hamata* which transports Mn^{2+} into the vacuole (Delhaize 2003).

NRAMP proteins are transition metal cation/proton co-transporters or antiporters with broad specificity (Cailliatte et al. 2009). AtNRAMP1 is a high affinity Mn uptake transporter. NRAMP5 is responsible for Mn and Cd uptake in rice (Sasaki et al. 2012). AtNRAMP3 and AtNRAMP4 play a key role in iron nutrition of the germinating plantlet by remobilizing vacuolar iron. The Zn and Cd hypersensitivity of *nramp3 nramp4* double mutants is likely to be a result of impaired remobilization of Fe from the vacuole. AtNRAMP6, which is targeted to a vesicular-shaped endomembrane compartment distinct from the vacuole or mitochondria, increases sensitivity to Cd without affecting Cd content. The null allele of NRAMP6 was more tolerant to Cd (Cailliatte et al. 2009).

Eight *A. thaliana* YSL oligopeptide transporters are expected to import transition metals complexed with NA into the cytosol (Schaaf 2005). AtYSL2 is expressed in cells around vascular tissues and translocates Cu(II)–NA and Fe(II)–NA complexes, which suggests its function in metal export from the vasculature. AtYSL2 transcript abundance decreases in shoots in response to Fe deficiency and Cu excess. AtYSL1 is expressed in the xylem parenchyma of leaves, where it is induced in response to Fe excess; ysl1 mutants accumulate more NA in shoots and less Fe and NA in seeds, suggesting that YSL1 participates in iron delivery to seeds (Le Jean et al. 2005). AtYSL1 and AtYSL3 show the highest expression in senescing rosette and cauline leaves. The double mutant ysl1ysl3 exhibited Fe deficiency and elevated concentrations of Cu, Mn and Zn (Waters et al. 2006). The *N. caerulescens* YSL3

is a Fe–NA influx transporter at pH = 7.0 and Ni–NA transporter at pH = 5.0 (Gendre et al. 2006).

Two ABC transporters, AtABCC1 and ABCC2, have been shown to contribute to transport of As–, Cd– and Hg–phytochelatin complexes into the vacuole. Simultaneous overexpression of AtABCC1 with AtPCS1 (phytochelatin synthase) resulted in plants exhibiting an increased arsenic tolerance (Song et al. 2010b). *A. thaliana* vacuolar membrane major facilitator superfamily protein ZIF1 gene was selected by genetic screening for Zn-hypersensitive mutants (Haydon and Cobbett 2007). ZIF1 overexpression enhances NA and Zn partition into and accumulation in vacuoles and impaired Fe movement and Fe deficiency symptoms (Haydon et al. 2012). *A. thaliana* Zn-efflux transporter PCR2, located in the epidermis and xylem of young roots, and in the epidermis of fully developed roots, contributes to root-to-shoot Zn transport. The pcr2 mutants are sensitive to both limitation and excess of Zn, and pcr2 roots accumulate more Zn than WT, and in Zn limiting conditions Zn is accumulated in the epidermis in pcr2, while in WT it is found in the stele (Song et al. 2010a). *A. thaliana* mutants exhibiting As(V) tolerance harbour null alleles coding for the high affinity Pi transporters PHT1;1 or PHT1;4, indicating that these transporters play a major role in As(V) uptake. Additionally, PHF1 (PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1), which is required for efficient trafficking of Pi transporters to the plasma membrane, also results in a strong tolerance to As(V) (Gonzalez et al. 2005). *A. thaliana* pht1;1 displays a slow rate of As(V) uptake that ultimately enables the mutant to accumulate double the arsenic found in wild-type plants. In *A. thaliana* As(V) represses the activation of genes involved in phosphate uptake, which may reflect a regulatory mechanism which protects plants from As uptake (Catarecha et al. 2007).

Arsenite As(III) uptake and translocation are mediated by members of the NIP subfamily of aquaporins (aquaglyceroporins) having a larger pore size; thus it is permeable for additional substrates, such as neutral metalloids, undissociated acids and small solutes like glycerol (Ali et al. 2009). Three independent As(III)-tolerant mutants were isolated from ethyl methanesulfonate-mutagenized seeds of *A. thaliana*; all mutations were located in the Nodulin 26-like intrinsic protein 1;1 (NIP1;1) gene. NIP1;1 is localised to the plasma membrane and is highly expressed in roots. Disruption of NIP1;1 function confers As(III) tolerance to plants and lowers As accumulation. NIP1;2 and NIP5;1, closely related homologues of NIP1;1, were also permeable to As(III). Disruption of these genes also reduced the As content in plants, but As(III) tolerance was not observed in nip1;2 and nip5;1 mutants (Kamiya et al. 2009). The fern *Pteris vittata* hyperaccumulates arsenic up to >1 % of the dry weight of a frond, 25 times more than in the root. Two *P. vittata* genes, ACR3 and ACR3;1, encode proteins similar to the ACR3 arsenite effluxer of yeast. ACR3 localises to the vacuolar membrane and its transcription is induced by arsenic in tissues that directly contact soil. It has been suggested that ACR3 may participate in transporting arsenic from the root into the xylem for translocation to the shoot (Indriolo et al. 2010).

3.4.3 Comparative Transcriptomics in Hyperaccumulator Gene Discovery

The comparative analyses of transcription profiles of closely related hyperaccumulator and non-accumulator plants, wild-type (WT) plants with insertion mutants of the same cultivar or plants with the same genetic background grown on media containing different meta(loid) concentrations, led to the detection of hundreds of differentially regulated "candidate genes". In several cases, the biological function of a candidate gene can be predicted and further tested; however, the function of a huge majority of these candidates cannot be easily predicted, so they have not yet been tested. Cadmium treatment affects regulation of a broad range of *A. thaliana* genes in several hours. Many genes involved in glucosinolate biosynthesis and photosynthesis are repressed, while genes involved in sulphur uptake and assimilation or cell wall and phenylpropanoid metabolism are induced, and indeed in *A. thaliana* sulphur uptake increases rapidly upon Cd treatment (Herbette et al. 2006). *A. thaliana* low affinity plasma membrane nitrate transporter NRT1.8 is expressed in xylem parenchyma cells and is induced by Cd stress. Disruption of the NRT1.8 gene shows that NTR1.8 takes up nitrate from xylem vessels to parenchyma cells and that nitrate allocation to roots is important for Cd tolerance (Li 2010). Unfortunately, several other genes important for Cd tolerance, such as phytochelatin synthase AtPCS1 or transporters ABCC1 and ABCC2, are expressed constitutively (Song et al. 2010b).

Roots of *A. halleri* grown in normal conditions revealed much higher constitutive expression of NAS and Zn transporters ZIP9 and NRAMP3. Comparison of shoot transcriptomes after exposure to low or high Zn revealed higher transcriptional induction of over 50 genes in *A. halleri* than in *A. thaliana* in both treatments, among them Zn transporters (HMA3, ZAP, ZIP6, CDF1), NAS and AGO5—a microRNA binding protein (Becher et al. 2004). Transcriptomes of whole plants grown under control conditions and upon short-term exposure to high Zn concentrations, compared using ATH1 microarrays, revealed further candidate transporter genes, induced more strongly in *A. halleri* than in *A. thaliana*, such as HMA4, IRT3 and ZIP10 (Talke et al. 2006). Function of the huge majority of these candidate genes in metal(loid) tolerance and hyperaccumulation remains to be elucidated. RNAseq-based transcriptomics provides more detailed information about gene expression and better transcriptome coverage than microarrays. Using this technology Bernal et al. (2012) have recently discovered that copper uptake depends on Cu(II) reduction to Cu(I) by FRO4/FRO5 (Bernal et al. 2012).

3.5 Conclusion

Prospects for the development of phytoremediation may stem from almost all presented aspects; however, it is certain that the future of this method may be associated with highly specialised plants (probably after complex genetic

modifications), which will possess most traits of plants suitable for phytoremediation. Our knowledge about the theoretical basis of biochemical plant response and genetic traits in response to presence of metals in connection with practical experiments will be one of the most significant factors affecting fast development of phytoremediation. Apart from the plant itself, it will also be essential to properly adapt plants to substrate conditions, moisture content as well as their ready applicability. It is evident that in the search for new strains of bacteria and fungi the above described objectives may be attained, in this way contributing to an increase of plant biomass, which will constitute a significant factor in the selection of the decontamination method of polluted soil. Due to the simultaneously conducted work on biological and technical methods, we may expect successive studies combining both types of methods. Such a combination will make it possible to significantly reduce costs of the method connected with the application of technical measures and at the same time will facilitate a shortening of time needed to attain required effectiveness of the soil remediation process. The last, but not the least concern is associated with the issue constituting a favourite argument for opponents of biological methods, which is the claim concerning the impossibility of further utilisation of plant materials containing considerable amounts of heavy metals. It may be assumed that such methods as phytomining or combustion of contaminated biomass in specially designed furnaces may solve this problem.

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Chapter 4
Remediation Mechanisms of Tropical Plants for Lead-Contaminated Environment

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4.1 Introduction

Heavy metals are a unique class of toxicants since they cannot be broken down to nontoxic forms. Concentration of these heavy metals has increased drastically, posing problems to health and environment, since the onset of the industrial revolution. Once the heavy metals contaminate the ecosystem, they remain a potential threat for many years (Jabeen and Ahmad 2012). The threat of heavy metals to human and animal health is aggravated by their long-term persistence in the environment (Gisbert et al. 2003). For instance, Pb, one of the more persistent metal, was estimated to have soil retention time of 150–5,000 years and was reported to maintain high concentration for as long as 150 years after sludge application to soil (Nanda Kumar et al. 1995).

Lead (a chemical element with symbol Pb) is a silvery-white highly malleable metal. Among its physical properties, at normal environmental conditions this metal is presented in the solid state and is dense, ductile, and very soft with poor electrical conductivity when compared to most other metals. The chemical symbol for lead (Pb) is an abbreviation of the Latin word plumbum, meaning soft metal. Pb is rarely found in native form in nature, but it combines with other elements to form a variety of interesting and beautiful minerals. Galena, which is the dominant Pb ore mineral, is blue-white in color when first uncovered but tarnishes to dull gray when exposed to air. Archeological research indicates that Pb has been used by humans for a variety of purposes for more than 5,000 years. In fact, archeological discoveries found glazes on prehistoric ceramics. The Egyptians used grounded

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Pb ore as eyeliner with therapeutic properties and cosmetic kohl; Pb-based pigments were used as part of yellow red and white paint. In ancient Rome, Pb was used to build pipes for water transportation (Rehren 2007).

Plants are the target of a wide range of pollutants that vary in concentration, speciation, and toxicity. Such pollutants mainly enter the plant system through the soil (Arshad et al. 2008) or via the atmosphere (Uzu et al. 2010). Among common pollutants that affect plants, lead is one of the most toxic and frequently encountered (Cecchi et al. 2008; Grover et al. 2010; Shahid et al. 2011). Lead continues to be used widely in many industrial processes and occurs as a contaminant in all environmental compartments (soils, water, the atmosphere, and living organisms). The prominence of environmental lead contamination results both from its persistence (Islam et al. 2008; Andra et al. 2009; Punamiya et al. 2010) and from its present and past numerous sources. These sources have included smelting, combustion of leaded gasoline, or applications of lead-contaminated media (sewage sludge and fertilizers) to land (Piotrowska et al. 2009; Gupta et al. 2009; Sammut et al. 2010; Grover et al. 2010). In 2009, production of recoverable lead from mining operations was 1,690, 516, and 400 thousand metric tons by China, Australia, and the USA, respectively. Despite a long history of its beneficial use by humankind, lead has no known biological function in living organisms (Maestri et al. 2010) and is now recognized as a chemical of great concern in the new European REACH regulations (EC 1907/2006; Registration, Evaluation, Authorization, and Restriction of Chemicals). Moreover, lead was reported as being the second most hazardous substance, after arsenic, based on the frequency of occurrence, toxicity, and the potential for human exposure by the Agency for Toxic Substances and Disease Registry (ATSDR 2003). The transfer of lead from polluted soils to plants was therefore widely studied, especially in the context of food quality use in phytoremediation, or in bio-testing (Arshad et al. 2008; Uzu et al. 2009). Lead is known to induce a broad range of toxic effects to living organism, including those that are morphological, physiological, and biochemical in origin. This metal impairs plant growth, root elongation, seed germination, seedling development, transpiration, chlorophyll production, lamellar organization in the chloroplast, and cell division (Sharma and Dubey 2005; Krzeslowska et al. 2009; Gupta et al. 2009, 2010; Maestri et al. 2010). However, the extent of these effects varies and depends on the lead concentration tested, the duration of exposure, the intensity of plant stress, the stage of plant development, and the particular organs studied. Plants have developed various methods for responding to toxic metal exposures. They have internal detoxification mechanisms to deal with metal toxicity that includes selective metal uptake, excretion, and complexation by specific ligands, and compartmentalization (Gupta et al. 2009; Krzesłowska et al. 2010; Maestri et al. 2010; Singh et al. 2010; Jiang and Liu 2010). The various responses of plants to lead exposure are often used as tools (bio indicators) in the context of environmental quality assessment.

4.2 Phytoremediation

Traditionally techniques of soil remediation are costly and may cause secondary pollution. Phytoremediation is an evolving field of science and technology to clean up polluted soil, water, or air. It may be defined as the use of green plants to remove, destroy, or sequester hazardous substances from the environment. Plants that uptake heavy metals from the soil offer an alternative and less expensive method to strip heavy metals directly from the soil. Plants have constitutive and adaptive mechanisms for accumulating or tolerating high contaminant concentrations in their rhizospheres (Yang et al. 2005). Phytoremediation takes advantage of the fact that a living plant acts as a solar-driven pump, which can extract and concentrate certain heavy metals from the environment (Raskin et al. 1997). Phytoremediation can provide a cost-effective, long-lasting aesthetic solution for remediation of contaminated sites (Ma et al. 2001). It maintains the biological properties and physical structure of the soil (Yang et al. 2005). One of the strategies of phytoremediation of metal-contaminated soil is phytoextraction, i.e., uptake and accumulation of metals into plant shoots, which can then be harvested and removed from the site. Another application of phytoremediation is phytostabilization where plants are used to minimize metal mobility in contaminated soils. Plant metal uptake is influenced by soil factors including pH, organic matter, and cation exchange capacity as well as plant species, cultivar, and age. The mobility and availability of heavy metals in soil are generally low, especially when soil is high in pH, clay, and organic matter (Jung and Thornton 1996; Rosselli et al. 2003). It is important to use the native plants for phytoremediation because these plants are often better in terms of survival, growth, and reproduction under environmental stress than plants introduced from other environments. There has been a continuing interest in searching for native plants that are tolerant to heavy metals; however, studies have evaluated the phytoremediation potential of native plants under field conditions (Shu et al. 2002; McGrath and Zhoa 2003; Abioye et al. 2012). Heavy metals can cause severe phytotoxicity and may act as powerful force for the evolution of tolerant plant populations. Therefore, it is possible to identify metal-tolerant plant species from natural vegetation in the field sites that are contaminated with various heavy metals. Hyperaccumulators which are often found growing in polluted areas can naturally accumulate higher quantities of heavy metal in their shoots than roots. In view of this fact, metal removal from soil can be greatly enhanced by the judicious selection of plant species; the knowledge about the ability of various plant species or tissues to absorb and transport metals will provide an insight into choosing appropriate plants for phytoremediation (Deng et al. 2004; Zhou and Song 2004). Identification of hyperaccumulators is an imperious and important task as the key to successful implementation of phytoremediation (Zhou 2002; Zhou and Song 2004). The hyperaccumulators characterized at first were members of the *Brassicaceae* and *Fabaceae* families (Salt et al. 1998). Presently at least 45 families are known to contain metal accumulating species. To date, more than 400 plant species of metal hyperaccumulator plants have been reported in the

literature (Salt et al. 1998). Hyperaccumulation of metals has been found in temperate as well as tropical regions throughout the plant kingdom, but is generally restricted to endemic plant species growing on mineralized soil and related rock types (Baker et al. 1989).

Heavy metal contamination of the soil has become serious and continuous problem of the world, which has attracted a great deal of attention from government and regulatory authorities in the past few decades to prevent further heavy metals' addition and soil deterioration and to implement possible methods of remediation (Ahmad et al. 2011). Humans and ecosystem may be exposed to chemical hazards such as heavy metals (lead, chromium, arsenic, zinc, cadmium, copper, mercury, and nickel) through the direct ingestion of contaminated soils, consumption of crops and vegetables grown on the contaminated lands, or drinking water that has percolated through such soils (McLaughlin et al. 2000). For example, in their assessment, Chaney et al. (2005) indicated that subsistence farmers eating rice grain grown on contaminated sites throughout their lifetime are at risk from dietary exposure to cadmium. With greater awareness by the governments and the public of the implications of degraded environment on human and animal health, there has been increasing interest amongst the scientific community in the development of technologies to remediate contaminated sites (Bolan et al. 2008). In developing countries with great population density and scarce funds available for environmental restoration, low-cost and ecologically sustainable technologies are required to remediate contaminated lands so as to reduce the associated risks, make the land resource available for agricultural production, enhance food security, and scale down land tenure problems. Remediation of heavy metal-contaminated sites is particularly challenging because unlike organic contaminants which are oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation and are toxic and their total concentration in soils persists for a long time after their introduction (Adriano 2003; Kirpichtchikova et al. 2006). Remediation techniques include (1) ex situ (excavation) or in situ (on-site) soil washing/leaching/flushing with chemical agents, (2) chemical immobilization/stabilization method to reduce the solubility of heavy metals by adding some nontoxic materials into the soils, (3) electro kinetics (electro migration), (4) covering the original polluted soil surface with clean soils, and (5) dilution method (mixing polluted soils with surface and subsurface clean soils to reduce the concentration of heavy metals).

Forms of lead include ionic lead (Pb^{2+}), lead oxides, and hydroxides, and lead–metal oxyanion complexes are the general forms of lead that are released into the soil, groundwater, and surface waters. The most stable forms of lead are Pb^{2+} and lead-hydroxy complexes. Pb^{2+} is the most common and reactive form of lead, forming mononuclear and polynuclear oxides and hydroxides (Ground-Water Remediation Technologies Analysis Center 1997). The predominant insoluble lead compounds are lead phosphates, lead carbonates (form when the pH is above 6), and lead hydroxides (Raskin and Ensley 2000). Lead sulfide (PbS) is the most stable solid form within the soil matrix and forms under reducing conditions when

increased concentrations of sulfide are present. Under anaerobic conditions a volatile organolead (tetramethyl lead) can be formed due to microbial alkylation (Ground-Water Remediation Technologies Analysis Center 1997).

4.2.1 Lead Phytotoxicity

Lead is known to negatively affect some of the most classical end points of plant toxicity like seed germination rate, seedling growth, dry mass of roots and shoots, photosynthesis, plant water status, mineral nutrition, and enzymatic activities (Munzuroglu and Geckil 2002). In general, effects are more pronounced at higher concentrations and continuance. In some cases, lower concentrations can stimulate metabolic processes and the enzymes involved in those processes (Gomes 2011). These negative effects can be expressed as symptoms in the form of chlorotic spots, necrotic lesions in leaf surface, senescence of the leaf, and stunted growth. Germination of seeds is drastically affected at higher concentrations. Development and growth of root and shoot in seedling stage are also affected. Pb negatively influences growth by reducing the uptake and transport of nutrients in plants, such as Ca, Fe, Mg, Mn, P, and Zn, and by blocking the entry or binding of the ions to ion carriers making them unavailable for uptake and transport from roots to leaves (Xiong 1997). Thus, Pb interferes with several physiological and biochemical processes of plant (Gomes 2011). Many European countries have adopted a bioavailability-based rationale to improve the reliability of assessments of metal uptake (Prueb 1997). Current legislation in most countries still uses total soil metal concentration as a simple index of hazard in contaminated soils, even though this approach does not take into account of soil characteristics which influence the bioavailability of metallic pollutants in contaminated soil (Datta and Young 2005).

4.2.2 Lead Uptake by Plants

With the exception of the special conditions that exist for plants cultivated near metal recycling industries (Uzu et al. 2010), the main pathway by which plants accumulate metals is through root uptake from soils (Fig. 4.1) (Sharma and Dubey 2005; Uzu et al. 2009). Part of the lead present in the soil solution is adsorbed onto the roots, and then becomes bound to carboxyl groups of mucilage uronic acid, or directly to the polysaccharides of the rhizoderm cell surface (Seregin and Ivanov 2001). Lead adsorption onto roots has been documented to occur in several plant species: *Vigna unguiculata* (Kopittke et al. 2008), *Festuca rubra* (Ginn et al. 2008), *Brassica juncea* (Meyers et al. 2008), *Lactuca sativa* (Uzu et al. 2009), and *Funaria hygrometrica* (Krzesłowska et al. 2009, 2010). Once adsorbed onto the rhizoderm root surface, lead may enter the roots passively and follow translocating water streams. However, lead absorption is not uniform along plant roots as a lead

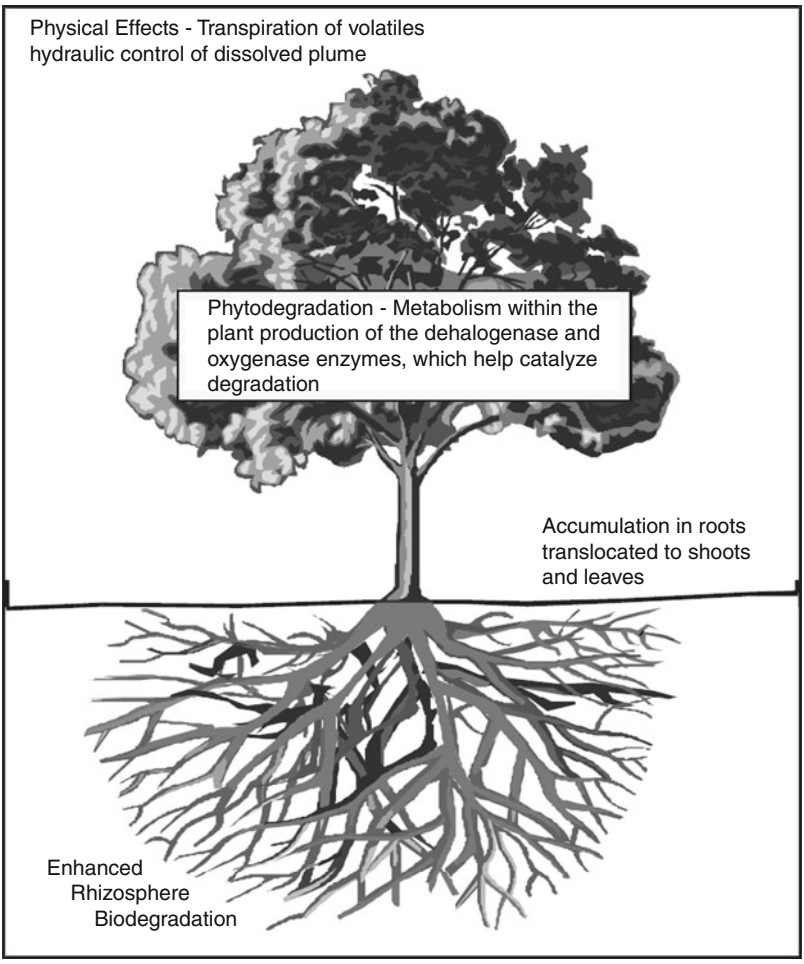

Fig. 4.1 Mechanism for phytoremediation (Source: United States Environmental Protection Agency 2000)

concentration gradient from root apex can be observed (Tung and Temple 1996; Seregin et al. 2004). Indeed, the highest lead concentrations can be found in root apices, where root cells are young and have thin cell walls (with the exception of root cap cells) that facilitate root uptake (Tung and Temple 1996; Seregin et al. 2004). Moreover, the apical area is the area where rhizodermic pH is the lowest, which increases solubility of lead in the soil solution. At the molecular level, the mechanism by which lead enters roots is still unknown. Lead may enter the roots through several pathways, and a particular pathway is through ionic channels. Although lead uptake is a nonselective phenomenon, it nonetheless depends on the functioning of an H+/ATPase pump to maintain a strong negative membrane potential in rhizoderm cells (Hirsch et al. 1998; Wang et al. 2007). Inhibition of lead

absorption by calcium is well known (Garland and Wilkins 1981; Kim et al. 2002) and is associated with competition between these two cations for calcium channels (Huang and Cunningham 1996). Several authors have demonstrated that Ca^{2+}-permeable channels are the main pathway by which lead enters roots (Wang et al. 2007; Pourrut et al. 2008). The use of transgenic plants has shown that lead can penetrate into roots through alternative nonselective pathways, such as cyclic nucleotide-gated ion channels (Arazi et al. 1999; Kohler et al. 1999), or via low-affinity cation transporters (Wojas et al. 2007).

Reduced uptake and translocation of lead to aerial parts of vegetables is considered to be beneficial in preventing lead from entering the food chain. However, reduced uptake and translocation of lead to aerial plant parts, when plants are used to remediate polluted soils, is a major problem. Indeed, soil remediation requires plants (hyperaccumulators) that can take high lead levels up and translocate it to aerial plant parts with no or minimal toxicity. The amount of lead that moves from soil to penetrate into plants can be measured by the transfer factor; transfer factor is defined as the ratio that exists between the concentration of lead in the plant vs. the concentration of lead in the soil (Arshad et al. 2008; Bi et al. 2010; Liu et al. 2010). Transfer factor will be different for different plant species and will change as soil's physical and chemical properties are altered (Arshad et al. 2008; Bi et al. 2010; Liu et al. 2010). Generally, plants having a transfer factor greater than 1 are categorized as hyperaccumulators, whereas those with transfer factor less than 1 are termed as non-accumulators of lead (Arshad et al. 2008).

4.3 Tropical Plants Useful in Remediation of Lead

4.3.1 Phytoextraction of Lead by Tropical Plants

Certain tropical plants have been identified which have the potential to uptake lead. Many of these plants belong to the following families: Brassicaceae, Euphorbiaceae, Asteraceae, Lamiaceae, and Scrophulariaceae. *Brassica juncea*, commonly called Indian mustard, has been found to have a good ability to transport lead from the roots to the shoots, which is an important characteristic for the phytoextraction of lead (United States Environmental Protection Agency 2000a, b). The phytoextraction coefficient for Indian mustard (*Brassica juncea*) is 1.7 and it has been found that a lead concentration of 500 mg L^{-1} is not phytotoxic to this Brassica species (United States Environmental Protection Agency 2000a, b). A phytoextraction coefficient is the ratio of the metal concentration found within the surface biomass of the plant over the metal concentration found in the soil. Thus the greater the coefficient, the greater the uptake of contaminant (Fig. 4.2) (United States Environmental Protection Agency 2000). Some calculations indicate that *Brassica juncea* is capable of removing 1,550 kg of lead per acre *Thlaspi rotundifolium* sp. Cepaeifolium, a non-crop Brassica, commonly known as

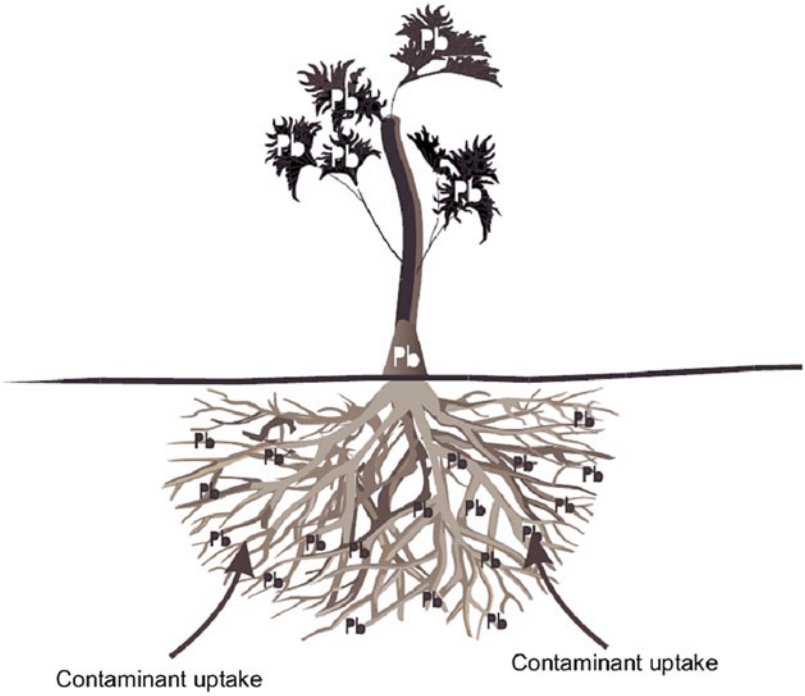

Fig. 4.2 Phytoextraction process of lead contaminant (Source: United States Environmental Protection Agency 2000)

Pennycress, has been found to grow in soils contaminated with lead (0.82 %) and zinc from a mine. Bench-scale studies have also shown that certain tropical plants are capable of phytoextraction. Corn, alfalfa, sorghum, cabbage, cauliflower, tomato, rice, barley, oats, wheat, corn, pigeon pea, chickpea, soybean, peanut, broccoli, lettuce, spinach, and amaranthus were found to be effective due to their fast growth rate and large amount of biomass produced (United States Environmental Protection Agency 2000).

4.3.2 The Roles of Sesbania drummondii *in the Phytoremediation of Lead*

Sesbania drummondii is one of potential tropical plants that can remediate lead-contaminated environment; it is a relatively large plant found growing naturally on a site contaminated with Pb, along with other inorganic and organic contaminants. For *Sesbania* to be useful in phytoremediation, it must not only accumulate large amounts of Pb from soil, but also translocate the Pb to aerial parts for harvest. Pb, however, is not very soluble in soil, and translocate poorly from roots to shoots

(Huang et al. 1997). One way to increase Pb solubility is to lower the pH. Decreasing the soil pH has also been shown to increase the amount of metal that a plant can accumulate (Huang et al. 1997). Alternatively, chelating agents such as EDTA have the potential to increase the bioavailability of metal in the soil solution and may increase the amount of metal accumulated in plant tissue (Huang et al. 1997).

4.4 Methods of Phytoremediation

Phytoremediation is actually a generic term for several ways in which plants can be used to clean up contaminated soils and water. Plants may break down or degrade organic pollutants, or remove and stabilize metal contaminants. This may be done through one of or a combination of the methods described.

4.4.1 Phytoextraction (Phytoaccumulation)

Phytoextraction is primarily used for the treatment of contaminated soils (United States Environmental Protection Agency 2000a, b). To remove contamination from the soil (Fig. 4.2), this approach uses plants to absorb, concentrate, and precipitate toxic metals from contaminated soils into the aboveground biomass (shoots, leaves, etc.) (Emerging Technologies for the Phytoremediation of Metals in Soil 1997). Discovery of metal hyperaccumulator species demonstrates that plants have the potential to remove metals from contaminated soils. A hyperaccumulator is a plant species capable of accumulating 100 times more metal than a common non-accumulating plant. Thus, a hyperaccumulator will concentrate more than 1,000 Fg g^{-1} (0.1 %) of Co, Cu, Cr, Pb, or 1 % of Zn and Ni in their leaf dry matter. Most hyperaccumulator species accumulate Ni while others have been shown to accumulate Cd, Co, Cu, and Zn. Currently there are no known Pb hyperaccumulators. Certain plants can extract lead from contaminated soils, but only when certain soil amendments have been added (United States Environmental Protection Agency 2000a, b). There are several advantages of phytoextraction. The cost of phytoextraction is fairly inexpensive when compared to conventional methods. For example, phytoremediation of an acre site contaminated with lead was estimated to require 30 years and cost $200,000 compared to $12 million for excavation and disposal, $6,300,000 for soil washing, and 600,000 for a soil cap (United States Environmental Protection Agency 2000a, b). Another benefit is that the contaminant is permanently removed from the soil (Emerging Technologies for the Phytoremediation of Metals in Soil 1997). In addition, the amount of waste material that must be disposed of is substantially decreased up to 95 % (United States Environmental Protection Agency 2000), and in some cases, the contaminant can be recycled from the contaminated plant biomass. The use of hyperaccumulator

species is limited by slow growth, shallow root system, and small biomass production. In addition, the plant biomass must also be harvested and disposed of properly, complying with RCRA standards (Emerging Technologies for the Phytoremediation of Metals in Soil 1997).

There are several factors limiting the extent of metal phytoextraction including:

- Metal bioavailability within the rhizosphere
- Rate of metal uptake by roots
- Proportion of metal "fixed" within the roots
- Rate of xylem loading/translocation to shoots
- Cellular tolerance to toxic metals

In order for this cleanup method to be feasible, the plants must extract large concentrations of heavy metals into their roots (Blaylock and Huang 1999), translocate the heavy metal into the surface biomass, and produce a large quantity of plant biomass (Brennan and Shelley 1999). In addition, the plants must have mechanisms to detoxify and/or tolerate high metal concentrations accumulated in their shoots (Brennan and Shelley 1999).

4.4.2 Phytostabilization

This is also referred to as in-place inactivation and is primarily used for the remediation of soil, sediment, and sludge (United States Environmental Protection Agency 2000). It is the use of plant roots to limit contaminant mobility and bioavailability in the soil (Emerging Technologies for the Phytoremediation of Metals in Soil 1997). The plants' primary purposes are to decrease the amount of water percolating through the soil matrix, which may result in the formation of a hazardous leachate (Blaylock and Huang 1999), act as a barrier to prevent direct contact with the contaminated soil, and prevent soil erosion and the distribution of the toxic metal to other areas (Raskin and Ensley 2000). Phytostabilization can occur through the sorption, precipitation, complexation, or metal valence reduction. It is useful for the treatment of lead (Pb) as well as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), and zinc (Zn) (United States Environmental Protection Agency 2000). Some of the advantages associated with this technology are that the disposal of hazardous material/biomass is not required (United States Environmental Protection Agency 2000), and it is very effective when rapid immobilization is needed to preserve ground and surface waters. The presence of plants also reduces soil erosion and decreases the amount of water available in the system (United States Environmental Protection Agency 2000). However, this cleanup technology has several major disadvantages including contaminant remaining in soil, application of extensive fertilization or soil amendments, mandatory monitoring is required, and the stabilization of the contaminants may be primarily due to the soil amendments (United States Environmental Protection Agency 2000). Phytostabilization has been used to treat contaminated land areas affected by

mining activities. Three grasses have been made commercially available after a field study conducted in Liverpool, England (Recent developments for in situ treatment of metals contaminated soil 1997):

- *Agrostistenuis*, *cv Parys* for copper waste
- *Agrostistenuis, cv Coginan* for acid lead and zinc wastes
- *Festucarubra, cv Merlin* for calcareous lead and zinc wastes

4.4.3 Rhizofiltration

Rhizofiltration is primarily used to remediate extracted groundwater, surface water, and wastewater with low contaminant concentrations. It is defined as the use of plants, both terrestrial and aquatic, to absorb, concentrate, and precipitate contaminants from polluted aqueous sources in their roots. Rhizofiltration can be used for Pb, Cd, Cu, Ni, Zn, and Cr, which are primarily retained within the roots (United States Environmental Protection Agency 2000).

Sunflower, Indian mustard, tobacco, rye, spinach, and corn have been studied for their ability to remove lead from water, with sunflower having the greatest ability. In one study, after only 1 h of treatment, sunflowers reduced lead concentrations significantly (Raskin and Ensley 2000). Indian mustard has a bioaccumulation coefficient of 563 for lead and has also proven to be effective in removing a wide concentration range of lead (4–500 mg L^{-1}) (Raskin and Ensley 2000). The advantages associated with rhizofiltration are the ability to use both terrestrial and aquatic plants for either in situ or ex situ applications. Another advantage is that contaminants do not have to be translocated to the shoots. Thus, species other than hyperaccumulators may be used. Terrestrial plants are preferred because they have a fibrous and much longer root system, increasing the amount of root area (Raskin and Ensley 2000). Disadvantages and limitations include the constant need to adjust pH; plants may first need to be grown in a greenhouse or nursery; there is periodic harvesting and plant disposal; tank design must be well engineered; and a good understanding of the chemical speciation/interactions is needed. The cost of remediation by rhizofiltration has been estimated to be $2–$6 per 1,000 gallons of water (United States Environmental Protection Agency 2000a, b).

4.5 The Mechanism of Tropical Plants in Remediation of Lead

4.5.1 Mechanisms of Lead Tolerance

Plants respond to noxious effects of lead in various ways, such as selective metal uptake, metal binding to the root surface, binding to the cell wall, and induction of antioxidants. There are several types of antioxidants to which plants may respond:

non-protein thiol (NP-SH), cysteine, glutathione, ascorbic acid, proline, and antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR). However, the response varies with plant species, metal concentration, and exposure conditions.

4.5.1.1 Chelates Assisted Mechanism in Phytoextraction of Lead

One major factor limiting the potential for lead phytoextraction is low metal bioavailability for plant uptake (Raskin and Ensley 2000). To overcome this limitation, synthetic chemical chelators may need to be added to the contaminated soil to increase the amount of lead that is bioavailable for the plants. The use of synthetic chelates in the phytoremediation process is not only to increase heavy metal uptake by plants through increasing the bioavailability of the metal, but also to increase micronutrient availability, which decreases the possibility of plant nutrient deficiencies (Blaylock and Huang 1999). The goal of commercial phytoextraction is to remove or reduce the level of toxic metals within the contaminated soils to meet regulatory standards within 1–3 years (Raskin and Ensley 2000). The regulatory standard for lead-contaminated soil set by the EPA is 500 ppm. Plants that accumulate more than 1 % of the target contaminant in the harvestable portion and produce more than 20 metric tons of shoot biomass per hectare per year are required to achieve this goal (Raskin and Ensley 2000). Researchers have found that through the application of soil amendments and chemical chelates this goal can be achieved. Based on scientific studies, it has been shown that only 0.1 % of the total amount of lead in contaminated soils is in solution and bioavailable to plants for remediation. With the addition of synthetic chelators, the total amount of lead in solution can be increased up to 100 times (Raskin and Ensley 2000). Increasing the mobility and bioavailability of lead in the soil through certain chelators, organic acids, or chemical compounds allows for the hyperaccumulation of metals in some plants. For lead, a number of different chelators have been tested: EDTA (ethylene-dinitrilo-tetra acetic acid), CDTA (*trans*-1,2-cyclohexylene-dinitrilo-tetra acetic acid), DTPA (diethylenetrinitrilo-penta acetic acid), EGTA (ethylebis[oxyethylenetrinitrilo]-tetra acetic acid), HEDTA (hydroxyethyl-ethylene-dinitrilo-tri acetic acid), citric acid, and malic acid. Addition of the chelates resulted in enhanced shoot lead concentrations. EDTA proved to be the best and least expensive, costing around $1.95 per pound. In soils with a pH of 5 and amended with EDTA, plants accumulated nearly 2,000 mg kg^{-1} more lead in their shoots when compared to other treatments in soil limed to a pH of 7.5. EDTA, DTPA, and CDTA all achieved shoot lead concentrations of more than 10,000 mg kg^{-1}.

In order for substantial lead accumulation (>5,000 mg kg^{-1}) to occur in the shoots, the concentration of synthetic chelates (EDTA, DTPA, and CDTA) must exceed 1 mol kg^{-1}. It was also noted that plants grown in soils amended with chelators varied in their lead concentration uptake. For example, the lead

concentration in peas (*Pisum sativum* L. cv Sparkle) was 11,000 mg kg^{-1} compared to corn, which accumulated 3,500 mg kg^{-1} in soils receiving equivalent amounts of EDTA (Malone et al. 1974). Although there are some advantages associated with the use of synthetic chelates, environmental concerns governing their impact on these contaminated sites are in need of research. The major concern associated with using chelates to enhance phytoremediation and increase the bioavailability of the toxic metals is the fear of lead leaching or running off into the ground or surface water. By making the metals more soluble in the soil matrix, leaching is more probable, threatening the contamination of nearby water sources (Reuther 1998).

4.5.1.2 Passive Mechanisms

Even when small amounts of lead penetrate root cell membranes, it interacts with cellular components and increases the thickness of cell walls (Krzesłowska et al. 2009, 2010). Pectin is a component of plant cell walls. Lead complexation with pectin carboxyl groups is regarded as the most important interaction by which plant cells can resist lead toxicity (Meyers et al. 2008; Jiang and Liu 2010). Krzesłowska et al. (2009) observed that binding of lead to JIM5-P (within the cell wall and its resultant thickening) acted as a physical barrier that restricted lead access to the plasma membrane in *F. hygrometrica* protonemata. However, later, these authors stated that lead bound to JIM5-P within the cell can be taken up or remobilized by endocytosis, together with this pectin epitope (Krzesłowska et al. 2010).

4.5.1.3 Inducible Mechanisms

Recently, several authors have reported the presence of transporter proteins among plant cells that play an important role in metal detoxification, by allowing the excretion of metal ions into extracellular spaces (Meyers et al. 2008; Vadas and Ahner 2009; Maestri et al. 2010). The human divalent metal transporter 1 (DMT1), expressed in yeast, has been shown to transport lead via a pH-dependent process in plants (Bressler et al. 2004). Simultaneously, several ATP-binding cassette (ABC) carriers, such as AtATM3 or AtADPR12 at ATP-binding sites in *Arabidopsis*, were involved in resistance to lead (Kim et al. 2006; Cao et al. 2008). Although suspected to act against lead, this detoxification mechanism has not yet been clearly confirmed. Transcriptome analysis has shown that the gene expression of these carriers is stimulated by lead (Liu et al. 2009). Cellular sequestration is considered to be an important aspect of plant metal homeostasis and plant detoxification of heavy metals (Maestri et al. 2010). The lead, which could be bound by certain organic molecules (Piechalak et al. 2002; Vadas and Ahner 2009), is sequestered in several plant cell compartments: vacuoles (Małecka et al. 2008; Meyers et al. 2008), dictyosome vesicles (Malone et al. 1974), endoplasmic reticulum vesicles (Wierzbicka et al. 2007), or plasma tubules (Wierzbicka 1998). Cysteine and glutathione (GSH) are known to be nonenzymatic antioxidants in plants.

An increase in cysteine content, in response to lead toxicity, has been demonstrated in *Arabidopsis thaliana* (Liu et al. 2009). Glutathione protects plants from lead stress by quenching lead-induced ROS (Verbruggen et al. 2009; Liu et al. 2009). Moreover, as the substrate for phytochelatin (PC) biosynthesis, the glutathione-related proteins play an important role in heavy metal detoxification and homeostasis (Liu et al. 2009). Lead treatment can induce different GSH genes, including glutathione-synthetase, -peroxidase, and -reductase, and glutamylcysteine synthetase. Glutathione can also enhance accumulation of proline in stressed plants, a role that is associated with reducing damage to membranes and proteins (Liu et al. 2009). Gupta et al. (2010) reported the role of GSH in lead detoxification in *S. alfredii,* although this was accomplished without any induction of PC. This suggests that GSH may play an important role in detoxifying lead, under stress conditions where PCs are absent. PCs and metallothioneins (MTs) are the best characterized metal-binding ligands in plant cells. These ligands belong to different classes of cysteine-rich heavy metal-binding protein molecules. PCs, the most frequently cited metal protective proteins in plants, are low-molecular-weight, metal-binding proteins that can form mercaptide bonds with various metals (Maestri et al. 2010) and play an important role in their detoxification in plants (Brunet et al. 2009; Liu et al. 2009; Gupta et al. 2010; Yadav 2010; Jiang and Liu 2010). These thiols are biologically active compounds, whose function is to prevent oxidative stress in plant cells (Verbruggen et al. 2009; Gupta et al. 2010). Their general structure is (γ-glutamyl-cys) *n* Gly where $n = 2–11$, and they are synthesized by the action of γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase; PCS) from GSH (Yadav 2010). Lead is known to stimulate the production of PC and activate PCS (Mishra et al. 2006; Clemens 2006; Andra et al. 2009; Vadas and Ahner 2009; Singh et al. 2010). It has been proposed that in vivo, phytochelatins are involved in the cellular detoxification and accumulation of several metals, including lead, because of their ability to form stable metal–PC complexes (Clemens 2006; Yadav 2010). Phytochelatin sequesters soluble lead in the cytoplasm before transporting it to vacuoles and chloroplasts (Piechalak et al. 2002; Małecka et al. 2008; Jiang and Liu 2010), thus reducing the deleterious effect of Pb^{2+} in the cells. The mechanism regulating the passage of the lead–PC complex through the tonoplast is, however, not yet known. Gisbert et al. (2003) reported significantly increased uptake and tolerance to lead and Cd following the induction and overexpression of a wheat gene encoding for phytochelatin synthase (*TaPCS1*) in *Nicotiana glauca*.

4.5.2 Antioxidant Enzymes

To cope with the increased production of ROS and to avoid oxidative damage, plants have a system of antioxidant enzymes that scavenge the ROS that are present in different cell compartments (Brunet et al. 2009; Singh et al. 2010; Gupta et al. 2010). Lead-induced toxicity may inhibit the activity of these enzymes or may

induce their synthesis. However, lead-induced inhibition or induction of antioxidant enzymes is dependent on metal type, specific form of the metal, plant species type, and the duration/intensity of the treatment (Islam et al. 2008; Gupta et al. 2009; Singh et al. 2010). Generally, lead inhibits enzymatic activities and, when this occurs, the values of the inactivation constant (K_i) range between 10^{-5} and 2×10^{-4} M (i.e., 50 % of enzymatic activities are inhibited in this concentration range) (Seregin and Ivanov 2001). Enzyme inhibition results from the affinity lead has for —SH groups on the enzyme (Sharma and Dubey 2005; Gupta et al. 2009). This is true for more than 100 enzymes, including ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and nitrate reductase. Inactivation results from a link at either the catalytic site or elsewhere on the protein and produces an altered tertiary structure. Lead can also produce the same effect by binding to protein—COOH groups (Gupta et al. 2009, 2010). Lead also interacts with metalloid enzymes. Indeed, lead can disrupt plant absorption of minerals that contain zinc, iron, manganese, etc., which are essential for these enzymes. Lead and other divalent cations also can substitute for these metals, and thereby inactivate enzymes, as occurs with ALAD (Gupta et al. 2009; Cenkci et al. 2010). The effect lead has on ROS constitutes another mechanism by which lead exposure affects protein behavior (Gupta et al. 2009, 2010).

4.6 Conclusion

Contamination of soil environment by lead is prevalent in developing countries and most industrialized countries as by-products of technology. Many techniques of remediating such contaminated soil have been developed. However, most of these methods have some drawbacks in terms of cost and efficiency. Phytoremediation with some selected tropical plants that possess hyperaccumulating potential for lead through different mechanisms of lead tolerance can offer a better and promising way of getting rid of lead from contaminated soil environment.

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Chapter 5
Impact of Metal/Metalloid-Contaminated Areas on Plant Growth

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5.1 Introduction

The development of industry, improvement of living conditions, and the use of traditional sources of energy have contributed to a drastic deterioration of the condition of the natural environment. Its overexploitation has caused soil and water pollution with several toxic and hazardous chemicals. Heavy metals and metalloids constitute a special group of pollutants due to their non-biodegradability as well as ready transport up the trophic chain. The problem of metals/metalloids, although usually limited to a given surface, is still global in character and should not be underestimated (Bhargava et al. 2012). Simple and relatively cheap remediation methods for degraded areas are searched for both in research papers and in industrial practice. Despite their limitations (Mench et al. 2010), currently applied biological methods (bioremediation and phytoremediation) are gaining popularity (Bone et al. 2010; Prasad et al. 2010). In the case of phytoremediation, growth of vegetation in a polluted area is frequently limited or even inhibited. In extreme cases we may observe plants withering immediately after planting or shortly afterwards. For this reason, it is a key element to select appropriate plants (species/variety), depending on their habitat requirements, adaptability, as well as characteristics of the polluted area (the type and concentration of pollutants, availability of water) (Shukla et al. 2011). The application of a specific plant in

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the remediation of degraded areas should be preceded by analyses in the hydroponic system and then field trials, in order to determine actual capacity to absorb toxic elements and to identify the effect of environmental conditions on plant behaviour (Zabłudowska et al. 2009). Another essential criterion is associated with biomass, since plants exhibiting high efficiency of heavy metal uptake are generally characterised by a slight increase in biomass (Hernández-Allica et al. 2008). In this respect full understanding of the genetic regulation of plant biomass production is crucial (Demura and Ye 2010). Within the last 20 years many different tools have been used to increase the efficiency of phytoremediation thanks to the application of specific additives in the form of complexing substances (Yan et al. 2012) and the application of bacteria.

5.2 Role of Soil Conditions in Natural Plant Growth (Macro- and Microelements)

Soil is a very important natural element of the environment preconditioning plant life. It covers the earth crust (lithosphere) with a thin stratum 1.5–2.0 m thick which developed from definite parent material under the influence of soil-forming factors and processes. The essence of these processes consists in transformations of mineral (degradation and synthesis) and organic (mineralisation and humification) materials, horizontal and vertical dislocation of many chemical elements and compounds, as well as exchange of matter and flow of energy between live organisms and soil substrate or soil phases (solid, liquid, and gaseous) (Prusinkiewicz 1999). The coloured effect of soil-forming processes involves appropriate formation and arrangement of genetic horizons in the soil perpendicular cross section. The upper horizons, called epipedons, as well as internal horizons referred to as endopedons, are characterised by strictly defined parameters and exert a strong impact on the membership of a given soil in a specific taxonomic unit (FAO 2007; PTG 2011).

From the point of view of plant growth and development as well as biomass production, near-surface horizons—frequently referred to as accumulative-humus horizons—play a particularly important role. They are made up of the following four components: mineral material and organic matter (solid phase), soil water (liquid phase), and air (gaseous phase) (Fig. 5.1). In mature soils, this triple-phase system remains in a certain state of dynamic equilibrium determining physical, chemical, and biological soil properties. A disturbance of this balance under the influence of natural factors is described as soil evolution, whereas any deterioration of soil properties as a result of anthropogenisation (human pressure) results in soil physical, chemical, or biological degradation. The most important constituents of the solid phase comprise mineral and organic colloids, i.e. particles of dimensions below 2 μM. Soils inherit their mineral colloid content together with their parent material from which they were formed. In the majority of cases, these are classic-sedimentary rocks and partially non-classic rocks, primarily, of post-glacial origin

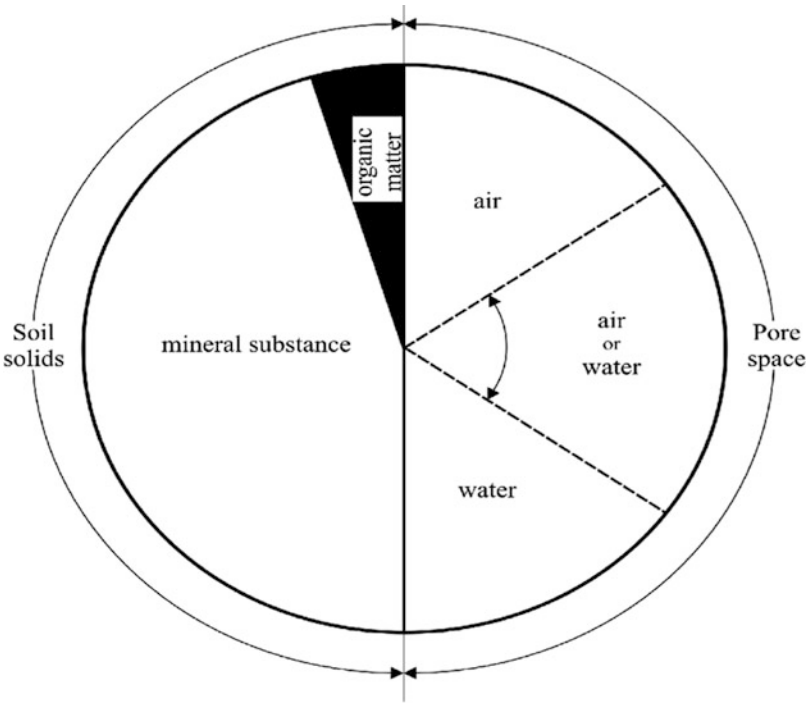

Fig. 5.1 Scheme of volume composition of mineral soil

(Mocek and Owczarzak 2011). Sources of organic colloids comprise substances mainly of plant but partly also of animal origin that find their way to soil and there they undergo mineralisation (about 70–80 %) and humification (about 20–30 %) processes. Mineralisation involves the breakdown of organic matter into simple mineral compounds such as CO_2, H_2O, and NH_3. Humification, on the other hand, is a process of transformation of organic residues in soil resulting in soil humus (Bednarek et al. 2004). Humus can be defined as a complex and relatively stable mixture of brown or dark-brown colloid substances (organic and organic-mineral) developed as a result of degradation of primary tissues, mainly of plant origin, and synthesis by various soil organisms (Brady 1990; Prusinkiewicz 1999).

Solid phase chemical composition exerts a decisive role regarding soil nutrient availability and not, as often wrongly claimed in the literature, soil fertility. Soil fertility is a much wider concept because it emphasises soil capability to meet edaphic requirements of various plants within the framework of possibilities created by the remaining site conditions (Prusinkiewicz 1999). Therefore, it may be concluded that fertile soil is a soil rich in nutrients (both macro- and microelements) and characterised by stable (appropriate) air–water parameters. In other words, every fertile soil must be rich in nutrients, but not every nutrient-rich soil can be considered as fertile. Mineral constituents essential for plants can be

found in soils in different forms, and different classifications can be found in the literature on this subject. The most frequent forms include (Filipek 2002):

- Active forms—constituents are found as ions or chelated molecules in soil solution
- Mobile forms—chemical elements as ions adsorbed by the soil sorptive complex
- Reserve forms—constituents as ions adsorbed in a non-exchangeable manner and forming part of crystalline structures of soil minerals

There is a definite dynamic equilibrium between all these forms, and quantitative and qualitative changes between individual chemical elements in the soil are shaped by physical, chemical, and biological processes taking place in the environment. Bearing in mind plant nutrient requirements, the following three groups of constituents of plant mineral nutrition are distinguished (Grzebisz 1996):

- Building—carbon, hydrogen, and oxygen
- Macroelements—nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur
- Microelements—iron, manganese, zinc, copper, boron, and molybdenum

Plants absorb nutrients from the soil solution mainly in the form of cations or anions. In addition, part of the constituents can be taken up in the form of chelates (many microelements) as well as organic molecules (e.g. in the form of urea, amino acids). A continuous exchange takes place between soil solution and the soil sorptive complex made up of mineral and organic colloids. This exchange, in soil science known as exchangeable sorption, refers primarily to cations, because soil colloid particles are in the majority negatively charged. Therefore, high concentrations of cations occur in the direct vicinity of the negatively charged surface of colloids at very low (close to zero) anion concentrations. This area is sometimes referred to as the Stern layer (Mengel and Kirkby 2001). Moving away from the colloid surface, numbers of cations decline, whereas those of anions increase slightly. This stratum, frequently called the Gouy-Chapman layer, is not very thick (5–10 nm) and passes into the soil solution, in which quantities of cations and anions are similar. A constant exchange also takes place between soil solution ions and the Gouy-Chapman stratum, and therefore, constituents taken up by plants from the soil solution are supplemented by diffused ions from the above-described double Gouy-Chapman stratum. Due to the fact that ions have different valences, the exchange occurs in a stoichiometric system, e.g. one magnesium cation is replaced by two cations of potassium.

Approximately 20 chemical elements are essential for appropriate plant growth and development. Some of them are absorbed in greater quantities and are called macroelements, of which the most important include N, P, K, Ca, Mg, and S. Other elements taken up by plants in small amounts are known as microelements (e.g. Fe, Mn, Cu, B, Zn, Mo, and Co). A synthetic collation of the most important plant nutritional components occurring in agroecosystem soils is presented in Table 5.1. From the point of view of biomass production, soil nutrient resources are most frequently regarded as total resources, in the majority inaccessible to plants; and

Table 5.1 Most important nutrients of crop plants in soils of agroecosystems (Filipek 2002) (slightly modified)

Nutrient	Form absorbed by plants	Total content and forms of occurrence	Occurrence of symptoms of	
			Shortage	Excess
Nitrogen (N) g kg^{-1}	NH_4^+, NO_3^-	0.2–6.0 (35.0), 98 % N org., 2 % N min.	All cultivated soils	In areas surrounding nitrogen processing enterprises, excessive N fertilisation
Phosphorus (P) g kg^{-1}	$H_2PO_4^-$, HPO_4^{2-}	0.1–1.5, 30–70 % P org., 30–70 % P min.	Strongly acid, alkaline soils	Not found
Sulphur (S) g kg^{-1}	SO_4^{2-}	0.01–0.8 (10.0), 95 % S org., 5 % S min.	Light soils	Industrial regions
Potassium (K) g kg^{-1}	K^+	8.0–25.0 %, soil minerals	Light, peat soils; alkaline earths	In conditions of liquid manure and slurry application
Calcium (Ca) g kg^{-1}	Ca^{2+}	3.0–16.0 (150.0), soil minerals	Light, strongly acid soils	Alkaline earths
Magnesium (Mg) g kg^{-1}	Mg^{2+}	0.5–6.0, soil minerals	Light, strongly acid soils	Not found
Sodium (Na) g kg^{-1}	Na^+	1.0−12.0 (20.0), soil minerals	Peat soils	Anthropogenic, seaside salty soils
Chlorine (Cl) g kg^{-1}	Cl^-	0.1–1.0 (20.0), ionic form in soil solution	Not found	Anthropogenic, seaside salty soils
Barium (B) mg kg^{-1}	$H_2BO_3^-$, H_3BO_3, $B_4O_7^{2-}$	7.0−40.0, soil minerals	Light, basic soils	Not found
Manganese (Mn) g kg^{-1}	Mn^{2+}, chelates	13.0–200.0, soil minerals	Alkaline soils in oxidative conditions	Acid soils, reductive conditions
Copper (Cu) mg kg^{-1}	Cu^{2+}, chelates	1.0−111.0	Organic soils	Industrial areas
Zinc (Zn) mg kg^{-1}	Zn_2^+, $Zn(OH)^+$, chelates	5.0–362.0, soil minerals	Alkaline soils	Industrial areas
Molybdenum (Mo) mg kg^{-1}	MoO_4^{2-}	0.1–11.0	Light soils	Not found
Iron (Fe) g kg^{-1}	Fe^{2+}, Fe^{3+}, chelates	0.2–40.0	Alkaline soils, oxidative conditions	Acid soils, reductive conditions

available resources, referring to the amounts of macro- and microelements which can be absorbed from the soil solution and, partially, also from the sorptive complex or some poorly soluble salts. The content of available forms in soils is determined most frequently using chemical laboratory methods. They are based on the preparation of extracts with the assistance of special, most often buffer, solutions and determination of a given component using spectrometric, colorimetric, or other methods. Next, the content of the specific macro- or microelement is compared with the boundary numbers which allow determination of the appropriate class of soil nutrient availability. Apart from many advantages, the basic disadvantage of these methods is lack of possibility of selection of universal extraction parameters (kind of solution, suitable proportions, extraction time, etc.) in different soils. Moreover, plants differ with respect to the structure of their root systems as well as different capabilities of nutrient absorption during various phases of development. In addition, also the prevailing soil conditions (moisture content, temperature, reaction, etc.) may affect this process.

For many centuries, for plant production purposes man used almost exclusively natural soil resources only partially supplemented by replenishment of the uptaken constituents in the form of natural and organic fertilisers. Hence, a definite state of equilibrium was maintained in soil resulting, on the one hand, from low levels of yields and, on the other, from a small mass of the components taking part in a traditional farming cycle (field–byre–field). Steady population increase made it necessary to intensify plant production through the application of new agrotechnical systems. This upset natural soil resources and made it unavoidable to introduce their supplementation by mineral fertilisation. These changes, in many instances, resulted in the occurrence of shortages or excess of soil nutrient constituents following the intensification of agricultural activities. In many places, the above problems were additionally aggravated by anthropogenisation of the environment (industrialisation, emissions of metal-bearing dusts, etc.). Moreover, plants can absorb some constituents in excessive quantities. This refers, in particular, to biogens such as nitrogen and sulphur. This often leads to the accumulation in plant tissues of amounts of these elements toxic to humans. This refers also to many microelements (boron, copper, zinc, manganese, molybdenum, and cobalt) which can be absorbed by plants in excessive quantities following abundant fertilisation by easily soluble compounds of the above-mentioned chemical elements.

Furthermore, it should be remembered that different compounds found in soils interact with one another, leading to the activation of some chemical elements and enhancement of their availability to plants. The phenomenon is known as synergism. Reverse phenomena, namely antagonisms, causing changes of easily available forms into ones which become unavailable to plants, also take place. Fertilisation with magnesium provides a good example of the above-mentioned phenomena as the increase of its content in soil increases zinc and manganese uptake but, simultaneously, limits absorption of potassium, calcium, and copper (Czuba 1996). Calcium is a cation exhibiting strongly antagonistic action in relation to other cations as well as to some anions. Liming, by changing soil reaction, abolishes a harmful effect of high quantities of aluminium and

manganese on plants. On the other hand, the excess of this cation in soil limits the availability of potassium, magnesium, iron, and, in particular, of zinc, manganese, and phosphorus. High soil copper content reduces possibilities of uptake by plants of iron, zinc, and manganese. In turn, zinc, manganese, and boron enhance the absorption of potassium and limit magnesium availability. In heavily limed soils, we observe low concentrations of boron in plant biomass, whereas shortages of molybdenum usually occur in plants cultivated on acid soils rich in iron (Czuba 1996). In the course of plant growth, it is possible to evaluate possibilities of their supply with nutrients contained in the soil in relation to the real state of nourishment by (Czuba 1996):

- Determination of plant available forms of macro- and microelements in the soil
- Determination of constituents in plants
- Observation of plants during different stages of their growth and development (identification of visible deficiencies)

Foliar application of fertilisers is one possible method of supplementation of the basic soil fertilisation. In such case, urea is used as a source of nitrogen, magnesium sulphate heptahydrate as a source of magnesium, and multi-component liquid fertilisers are applied as a source of many microelements.

5.3 Changes of Soil Parameters as Factors of Growth Stimulation or Inhibition

Apart from soil nutrient availability (macro- and microelements), there are a number of other soil parameters affecting plant growth and development or inhibition. They include properties that were inherited by soils from their parent material and which are relatively stable as well as anthropogenic properties resulting from geomechanical, hydrological, and chemical transformations. The most important soil parameters determining plant growth and development are physical parameters: soil texture, bulk density, porosity, as well as structure and water capacity. With respect to chemical parameters, the most important are content of organic matter, sorption, reaction, and buffer capacities. The most important, unchangeable soil character inherited from the parent material is soil texture. It refers to the size of individual soil grains (fractions) and their percentage composition in the soil solid phase. The most important role is played by clay fractions made up of clay minerals (silicates and aluminosilicates) which exert a decisive influence on nearly all soil characteristics. On the basis of texture, the following soils can be distinguished: sandy, loamy, clayey, and silty (Fig. 5.2). From among the abovementioned mineral soils, the most advantageous for the majority of crop plants are silty formations followed by loamy and clayey soils; sandy soils are the worst in this regard (Mocek and Drzymała 2010). Soil bulk density expresses the ratio of the solid phase to total soil volume. In the case of top layers of mineral soils, this parameter can fluctuate in the range of 0.9 to 1.9 g mL^{-1}, although most frequently

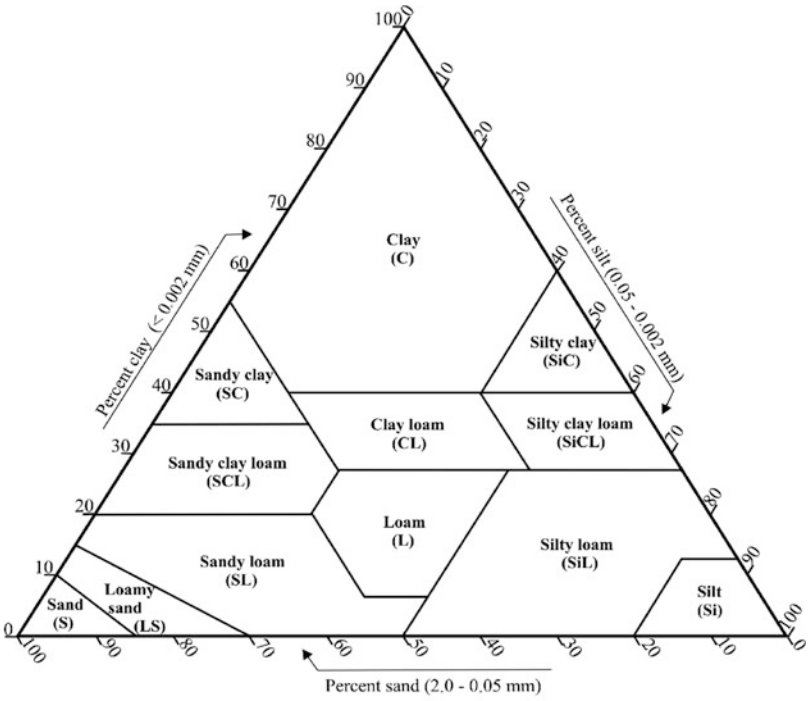

Fig. 5.2 Percentages of sand, silt, and clay in the major soil textural classes

it assumes the value of about 1.5 g mL^{-1} (Mocek and Drzymała 2010). Optimal values of this soil feature for selected crop plants are collated in Table 5.2. It can be concluded on the basis of the data presented in Table 5.2 that potatoes and sugar beets (only at the beginning of vegetation) have relatively weak root systems requiring extensive space for growth. Together with the increase of soil density, compaction of the surface soil layer takes place, which, once it exceeds 1.8 g mL^{-1}, makes growth and development of plant root systems impossible (Puchalski and Prusinkiewicz 1990). Soil density changes during the vegetation period, and as a rule, it increases with the passage of time, reaching a maximum at the beginning of autumn. Increasing soil compaction can be attributed to many factors, the most important of which include (Rząsa and Owczarzak 2004):

- Natural (gravitational) settling of soil after ploughing
- Compaction of soil during field preparation for sowing as well as in the course of performing agrotechnical operations
- Compacting action of soil water (mainly capillary)
- Ploughing at high soil water content
- Excessive proportion of root crop plants in the applied rotation system accompanied by lack of structure-forming plants

Table 5.2 Optimal soil density for some species of crop plants (Grzebisz 1996)

Crop plant	Bulk density (g mL^{-1})
Potato	1.0–1.2
Sugar beet	1.1–1.2
Winter wheat, light soil	1.4–1.6
Winter wheat, heavy soil	1.2–1.4
Barley, light soil	1.3–1.6
Barley, heavy soil	1.3–1.5
Oats	1.2–1.5
Maize	1.3–1.4
Flax	1.2–1.4

Soil density determines another soil property known as porosity, which expresses the total amount of all free spaces in a soil volume unit. In natural conditions, these spaces are occupied by water (liquid phase) and air (gaseous phase). The value of this trait in the surface layer of mineral soils can range from 30 to 75 %, but most frequently it assumes the value of 45–50 %. This feature is very unpredictable during the year and it also depends on soil origin and texture. Sometimes, minimal and maximal porosities are distinguished (Rząsa and Owczarzak 2004) and the interval between maximal and minimal porosity can be very wide. In general, soils developed from loesses are characterised by low and uniform (30–40 %) porosity values, while compact alluvial soils as well as rendzinas and clayey formations are distinguished by high (40–50 %) porosities. Boulder formations exhibit more uniform porosity intervals ranging from 35 to 40 % (Rząsa and Owczarzak 2004). Apart from general porosity, differential porosity is a very important soil characteristic expressing the proportions of pores of specific diameters. Different pore size boundaries are assumed, but as a rule, pores with the size of 8.5 μM are referred to as macropores, those with diameters ranging from 8.5 to 0.2 μM as mesopores, and those with sizes less than 0.2 μM as micropores. Optimal conditions for crop plant growth and development occur when macropores are filled with soil air, while meso- and micropores are saturated with water. Boundaries between liquid and gaseous phases are very labile and can assume different values during the vegetation period, accelerating or slowing down production of plant biomass.

Soil texture and porosity values exert a significant influence on the soil property referred to as soil structure. It expresses the size, shape, and degree of aggregation of the soil solid phase. For the growth and development of crop plants, aggregate structures (first and foremost, spheroidal ones) are most favourable and those having separate granular and coherent (massive) structures are much less advantageous (Brady 1990). The development of aggregates is strongly affected by the following factors:

- Wetting and drying
- Freezing and defreezing
- Physical action of roots and soil fauna

- Impact of decomposing organic matter as well as mucus excreted by micro-organisms and other living soil organisms
- Modifying impact of absorbed cations
- Soil cultivation

Quantitative and qualitative composition of the soil solid phase, through its porosity and structure, exerts a decisive influence on soil water retention capabilities. In this regard, the ability of soil to retain water available for plants, i.e. water held in soil mesopores, plays an exceptional role. In the overwhelming majority, it is capillary water whose quantities fluctuate between field water capacity (p*F* 2.0–2.5) and the point of permanent plant wilting (p*F* 4.2). It occurs as soil solution and moves from thicker to thinner films. The rate of plant root growth depends on the amount of water available to plants. In general, this growth is very fast, which allows plants good supply of water without greater participation of capillary forces (Buckman and Brady 1969). With respect to soil chemical properties, it is organic matter that exerts the strongest impact on plant growth and development. Its content in the surface layers of mineral soils can range from several decimal fractions to about 5 % (in soils in Poland, 2.0–2.5 %). Humus compounds constitute an important storage house of nutrients, primarily of nitrogen and phosphorus, which are liberated into the soil environment during the process of mineralisation. In addition, as the main constituent of the sorptive complex, the above-mentioned compounds retain many macro- and microelements which find their way into the soil solution as a result of exchangeable sorption and are utilised by plants. In the course of reactions with metal ions (Fe^{3+}, Al^{3+}, Ca^{2+}, and Mg^{2+}), humus compounds can form both simple and chelate complex compounds (Stevenson 1985; Ulrich 1983). In the case of strongly acid soils, humus plays a significant role in the detoxification of aluminium ions. Experiments conducted by Bloom et al. (1979) (after Bednarek et al. 2004) demonstrated that the addition of even small quantities of organic matter to a strongly acidified soil reduced the toxic effect of aluminium. This was also corroborated by investigations carried out by Myśków (1984), who reported that in soil containing 1 % humus, already 1 mg Al 100 g^{-1} soil resulted in a distinct yield reduction. In soils which contained about 5–6 % humus, a negative impact of aluminium was observed only at concentrations of 15 mg Al 100 g^{-1} soil.

According to Zaujec (2007), there is a rectilinear dependence between soil humus content and many physical soil properties, and, consequently, humus indirectly creates better conditions for plant growth and development. The increase of soil humus content by 0.1 % enhances water capacity by 0.5–0.6 gravimetric percentage, sorptive capacity by 0.7 cmol(+) kg^{-1}, and pore volume by about 1 %. Furthermore, it exerts a significant impact on peptide bonds by reducing their toxicity and accelerating sensitivity to biodegradation (Stevenson 1985). Papers were also published emphasising a direct impact of humic compounds on soil microorganisms and plants (Flaig 1975; Tołpa 1982). Certain fractions of these compounds can penetrate into plant roots creating reduction–oxidation (redox) systems inside cells and modifying their metabolism. Additionally, humus exerts a protective influence

on different biologically active compounds (vitamins, enzymes, phytohormones, etc.), extending their activity in soil (Bednarek et al. 2004).

Plant growth can be significantly repressed by mineral and organic xenobiotics finding their way to surface soil layers as a result of increasing anthropogenic pressure (Mocek and Mocek-Płóciniak 2011). These compounds include, primarily, heavy metals and polycyclic aromatic hydrocarbons (PAHs). By accumulating in plants, xenobiotics cause changes in their metabolism and induce mutagenic and carcinogenic changes in the next links of the trophic chain, namely among animals and humans. The inhibiting influence of the above-mentioned xenobiotics was also reported in both under- and aboveground parts of plants (Klimkowicz-Pawlas 2009; Smreczak and Maliszewska-Kordybach 2003). At small quantities of the above-mentioned pollutants (less than 100 mg kg^{-1} soil), a stimulating effect of PAHs on plant growth and development was sometimes observed (Klimkowicz-Pawlas 2009).

Soil, as a living formation, constitutes a natural environment for many different organisms. Micro- and mesoflora as well as micro- and mesofauna play a special role in making essential nutrients available to plants. Soil microorganisms perform almost all processes of biochemical nature which play a decisive role in processes of mineralisation and humification of residues of plant and animal origin. Huge quantities of microorganisms, ranging from 0.5 to 5 million bacteria and from 1,000 to 15,000 fungi, may be found in 1 g of soil (Smyk 1999). The total mass of such microorganisms inhabiting 1 ha of land can range from 1.5 to 15 mg. These enormous amounts of microbes, on the one hand, release nutrients indispensable for higher plants but, on the other hand, compete with them for the very same food. This refers, in particular, to nitrogen and to a lesser degree also to phosphorus, potassium, and calcium (Buckman and Brady 1969). Also the competition between microorganisms and plants is sometimes viewed negatively. The important trace elements indispensable as catalysers of plant physiological processes are usually taken up faster by microorganisms, which frequently results in shortages of these elements. Nevertheless, these inconveniences are more than compensated for by the advantageous impact of microorganisms on higher plants through processes of organic matter decomposition, transformation of mineral compounds, nitrogen fixation, etc. Measurements of soil enzyme activity are employed more and more frequently to assess the intensity of transformations taking place in soil under the influence of microorganisms. Dehydrogenases, phosphatases, ureases, and proteases turned out to be particularly useful in this regard (Bielińska 2001; Bielińska and Mocek-Płóciniak 2009). Soil enzymatic indices have become good indicators of soil biological condition or even an indirect way of determination of its fertility (Januszek 1999; Bielińska 2001). From the mesofauna group, earthworms exert a stimulating effect on plant growth and development. In 1 year, they can 'process' through their bodies about 35 mg of soil dry matter per hectare. This means that in approximately 70 years, the entire arable layer on an area of 1 ha passes through their organisms, resulting in enrichment of soil with numerous enzymes and addition of many nutrients, primarily nitrogen, essential for plant development and growth (Buckman and Brady 1969).

5.4 Phytoremediation Potential of Most Popular Plants

Heavy metals are conventionally defined as elements with metallic properties (ductility, conductivity, stability as cations, ligand specificity, etc.) and atomic number >20. The most common heavy metal contaminants are Cd, Cr, Cu, Hg, Pb, Co, Ni, and Zn. Metalloids are chemical elements with properties that are in between or a mixture of those of metals and non-metals and which are considered to be difficult to classify unambiguously as either a metal or a non-metal (B, Si, Ge, As, Sb, and Te). Both metals and metalloids are natural components in soil, but high levels resulting from industrial activities, such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertiliser and pesticide application, and generation of municipal waste (Kabata-Pendias and Pendias 1999), are the most serious environmental problems.

Some heavy metals and metalloids, such as As, Cd, Hg, or Pb, are not essential, since they do not perform any known physiological function in plants. Others, e.g. Co, Cu, Fe, Mn, Mo, Ni, and Zn, are essential elements required for normal growth and metabolism of plants. Heavy metal phytotoxicity may result from alterations of numerous physiological processes caused at the cellular/molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting for essential elements, and disrupting membrane integrity. A rather common consequence of heavy metal poisoning is the enhanced production of reactive oxygen species (ROS) due to interference with electron transport activities, especially those of chloroplast membranes (Pagliano et al. 2006; La Rocca et al. 2009; Rascioa and Navari-Izzo 2011). This increase in ROS exposes cells to oxidative stress, leading to lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage (Rascioa and Navari-Izzo 2011).

Plants respond with a series of defence mechanisms that control uptake, accumulation, and translocation of these dangerous elements and detoxify them by excluding the free ionic forms from the cytoplasm. Although all plants may extract toxic elements from soil, only some plants species may survive, grow, and reproduce under heavy metal/trace element contamination. What is interesting and very important is that these plants tolerate high concentrations of heavy metals, which are highly toxic for other species of plants. The identification of metal-accumulating plants has increased interest due to their use in remediation methods of contaminated soil. The technology of phytoremediation using hyperaccumulator plants to remove metals and contaminations from soils, sediments, and water by absorbing metals from soil, followed by their transport and accumulation in shoots, is called phytoextraction (Padmavathiamma and Li 2007; Van Nevel et al. 2007). The term hyperaccumulator was first applied during accumulation of nickel in *Sebertia acuminata* (Jaffre et al. 1976). The first definition of hyperaccumulator was plants that can accumulate more than 1,000 mg kg^{-1} Ni dry weight (dw) in their shoots (Brooks et al. 1977). Now hyperaccumulators are called species capable of exceptional accumulation of any kind of heavy metal in

shoots (100-fold greater level than those typically measured in common non-accumulator plants), i.e. more than 10,000 mg kg^{-1} dw for Ni, Zn, and Mn, 1,000 mg kg^{-1} dw for As, Pb, Cu, Ni, and Co, 100 mg kg^{-1} dw for Cd, 10 mg kg^{-1} dw for Hg, and 1 mg kg^{-1} for Au (Wei et al. 2008). To enhance metal uptake by plants, chelating agents and genetic manipulation are also used. Some hyperaccumulators have very unique ecophysiological behaviour and have the capacity to accumulate significant amounts of metals and compartmentalise them efficiently in the cell wall, vacuole, and to the specific subcompartment and/or compartments of the cytosol in order to render them innoxious or nontoxic and keep them away from active metabolic sites in plant cells (Memon and Schröder 2009).

Approximately 450 plant species from at least 45 plant families have been reported to hyperaccumulate metals (As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Se, Tl, and Zn). However, new reports of this kind of plants continue to accrue (Robinson et al. 2006; Sun et al. 2006; Venkatachalam et al. 2009; Rascioa and Navari-Izzo 2011), so it is conceivable that many yet unidentified hyperaccumulators may occur in the environment. As regards the many plants useful in decontamination of polluted areas and their different traits, it is important to present three selected plant groups (1) hyperaccumulators, (2) non-hyperaccumulators, and (3) transgenic plants.

5.4.1 Hyperaccumulators

5.4.1.1 *Thlaspi caerulescens* (Brassicaceae)

Possibly, *Thlaspi caerulescens* is the best-known metal hyperaccumulator. *T. caerulescens* (alpine pennycress) can accumulate Cd, Ni, Zn, and Pb. As a hyperaccumulator of Cd and Zn it could remove as much as 60 kg Zn ha^{-1} and 8.4 kg Cd ha^{-1} (Robinson et al. 1998). This plant species has an unusual ability not only to accumulate Pb in its roots but also to translocate Zn and Cd to the harvestable parts, as well as high rates of elements' uptake and translocation, and, what is more, the Pb ion is less accumulated in the shoots than in the roots. The species can be used for phytostabilization of Pb-contaminated sites (El Kheir et al. 2008). *T. caerulescens* has also been shown to develop a dense root system with a large proportion of fine roots, which may also contribute to enhanced uptake of metals (Keller et al. 2003; Wenzel 2009). What is more, *T. caerulescens* is useful for moderately Zn- and Cd-contaminated soils but would take far too long on highly contaminated ones. It also appears that season length, method of sowing seeds, and soil pH have effects on the capacity of *T. caerulescens* for extraction of Zn and Cd from the polluted soil (McGrath et al. 2006; Yanai et al. 2006). The efficiency of phytoextraction, besides biomass production, depends on the metal bio-concentration factor (BCF – the plant to soil concentration ratio) and for Zn and Cd it decreases log-linearly with soil metal concentration (Zhao et al. 2003). Moreover, the phytoremediation potential differs between different populations of *T. caerulescens*. The southern French ecotype showed a higher ability to

accumulate Cd than Zn; the different uptake of Cd and Zn shows that there are basic differences in the mechanism of accumulation of both metals in hyperaccumulators (McGrath et al. 2006). Thus, increased selection for traits of interest may help to improve the phytoremediation capacity of hyperaccumulators (McGrath et al. 2006). It is necessary to underline that also other species of *Thlaspi* have a good accumulation capacity and remediation capability: *T. goesingense* and *T. ochroleucum* hyperaccumulate Ni and Zn, while *T. rotundifolium* hyperaccumulates Ni, Pb, and Zn (Rascioa and Navari-Izzo 2011).

5.4.1.2 *Pteris vittata* (Pteridaceae) and *Reynoutria sachalinensis* (Polygonaceae)

The first defined arsenic hyperaccumulator, *Pteris vittata* L., was described by Ma et al. (2001). This plant may accumulate as much as 2.3 % arsenic in the fronds when grown in soil containing 1.5 g kg^{-1}, and is very efficient in removing arsenic from all fractions of the rhizosphere soil (Silva Gonzaga et al. 2006; Antosiewicz et al. 2008). The plants produce a relatively high biomass under favourable climate conditions, accumulating (from relatively low As concentration in the soil) 22 g As kg^{-1} in the frond dry weight, with a bioconcentration factor of 87 and a removal of 26 % of the initial As present in soil (Ma et al. 2001; McGrath and Zhao 2003). Other As-hyperaccumulating fern species have been identified more recently (Zhao et al. 2003). Great potential for phytoextraction has also been reported for *Reynoutria sachalinensis* (Polygonaceae). This plant accumulated As up to 1.9 g kg^{-1} dw in shoots and up to 0.43 g kg^{-1} dw in roots, and drastically reduced As concentrations in soil from up to 600 to 6–9 mg kg^{-1} dw after 5 years of cultivation and harvesting (Antosiewicz et al. 2008).

5.4.1.3 *Fagopyrum esculentum* (Polygonaceae)

Although Pb is largely an immobile element in soil and its extraction rate is limited by solubility and diffusion to the root surface, common buckwheat (*Fagopyrum esculentum*, Polygonaceae), the first known Pb hyperaccumulator species with high biomass, can accumulate up to 4.2 mg kg^{-1} dw of Pb in the shoots (Tamura et al. 2005). Amending the soil with the biodegradable methylglycine diacetic acid (MGDA) resulted in a fivefold increase in the Pb shoot concentration. This relevant finding qualifies this species as an excellent candidate for remediating Pb-contaminated soils.

5.4.1.4 *Phytolacca acinosa* (Phytolaccaceae)

This plant, which grows rapidly and has substantial biomass, has been considered to have potential for use in phytoremediation. The plant is able to accumulate Mn to 19.3 g kg^{-1} dw when grown on Mn-rich soils (Xue et al. 2004).

5.4.1.5 *Alyssum serpyllifolium* (Brassicaceae)

The efficiency of *Alyssum serpyllifolium* subsp. *lusitanicum* for use in phytoextraction of polymetal-contaminated soils has been examined (Kidd and Monterroso 2005). The plants have been grown on soils contaminated with Cr, Cu, Pb, and Zn. The results suggest that *A. serpyllifolium* can be suitable for phytoextraction in polymetal-polluted soils, provided that Cu concentrations are not phytotoxic. However, with the hyperaccumulators available, decades are needed to clean up contaminated sites.

5.4.1.6 *Alyssum bertolonii* (Brassicaceae) and *Berkheya coddii* (Asteraceae)

There are about 300 known species of Ni hyperaccumulators (Robinson et al. 1999), belonging to more than 33 families (Gramlich et al. 2011). Attractive plants for phytoextraction of Ni are *Alyssum bertolonii* (Gramlich et al. 2011) and *Berkheya coddii* (Moradi et al. 2010a). They are presented as effective plants on a commercial scale to extract Ni. *A. bertolonii* is a serpentine endemic nickel-hyperaccumulating plant with small biomass (Barzanti et al. 2011), while *B. coddii* has high phytoextraction potential and is characterised by high biomass (22 mg ha^{-1}) production (Moradi et al. 2010a). Unfortunately, there is little information about the mechanism of Ni hyperaccumulation or about root–soil interaction under Ni contamination (Moradi et al. 2010b). Knowledge and understanding of this issue could improve the results of phytoextraction methods for Ni.

5.4.1.7 *Noaea mucronata* (Chenopodiaceae)

N. mucronata is a typical dry desert shrub which grows in insufficient and poor soils (Pen-Mouratov et al. 2008). *N. mucronata* also belongs to the xerohalophytes and is the co-dominant plant over large areas. In recent years, the literature data have shown that among the native plants grown in the studied polluted area (north west of Iran), *N. mucronata* was found to be a good hyperaccumulator plant for Pb, Zn, and Cd (Chehregani et al. 2009; Parizanganeh et al. 2010). The amounts of heavy metals were decreased in polluted soils under the effect of *N. mucronata*, which makes them an effective accumulator, especially a good Pb accumulator (Chehregani et al. 2009).

5.4.2 Non-hyperaccumulators

Non-hyperaccumulators also have the possibility to accumulate significant amounts of heavy metals in plant organs. For example, plants inhabiting sites near old

arsenic/gold mines are exposed primarily to toxic concentrations of As, and to a lesser extent Pb, Al, and Fe. The use of a single extraction procedure demonstrated variations in the amount of arsenic released from the rhizosphere soil. This likely resulted from the species-specific root-induced modification of chemical forms of arsenic and its bioavailability, as a part of a plant strategy to survive in the contaminated environment. A plant species, *Calamagrostis arundinacea* (Poaceae), was identified which, due to its ability to substantially increase the arsenic concentration in the soil solution, likely by efficient uptake, reduced by 40 % the total As soil concentration in the root zone. The discovered case of natural phytoextraction points to the usefulness of this species for phytoremediation. Another plant species, *Stachys sylvatica* (Lamiaceae Lindl.), as a plant with low As concentrations in shoots and low concentration of bioavailable As in the rhizosphere (relative to other plants from the same area), was considered for further detailed study as a plant for possible use for phytostabilization. There were also species identified with exceptional ability to extract elements from the soil and accumulate them at high level in shoots:

1. Al by *Oxalis acetosella* (Oxalidaceae) and *Geranium robertianum* (Geraniaceae)
2. Mn by *Calamagrostis arundinacea* (Poaceae)
3. Fe, Sr, and Ba by *Fragaria vesca* (Rosaceae) (Antosiewicz et al. 2008)

Rubus ulmifolius (Rosaceae Juss.) never accumulated more than 1 g kg^{-1} of any of the metals in the aerial plant organs, the criteria indicated for As, Pb, or Ni hyperaccumulators (Marques et al. 2009). In fact, the metals were mainly accumulated in the roots of the plant, indicating a low metal translocation into the aerial section. Translocation rates between roots and stems ranged from 0.02 to 0.16 for As and from 0.08 to 0.13 for Pb. The high metal concentration in roots and apparent low translocation to the aerial plant organs indicate that the plant is capable of rather well-balanced accumulation and translocation (Haque et al. 2008). This may suggest a metal exclusion strategy from stems and reproductive tissue by retaining the metal in the roots (Marques et al. 2009), thus avoiding its toxicity. Resistance of *R. ulmifolius* to the metal's presence can be achieved by an avoidance mechanism such as precipitation and association with cell walls or detoxification in vacuoles (Marques et al. 2009). Although each plant species might have a unique mechanism against heavy metals, other published data also indicate higher accumulation of As (Madejón et al. 2003, 2007), Pb (Fitzgerald et al. 2003), and Ni in the roots of plants growing in metal-contaminated soils than in its aboveground tissues. *R. ulmifolius* also has shown the same type of accumulation behaviour for Zn; plants accumulated up to 563, 110, and 91 mg Zn kg^{-1} in the roots, stems, and leaves, respectively, for a level of Zn in the soil of up 957 mg kg^{-1} (Marques et al. 2009). This way of accumulation for all the studied metals truncates the biogeochemical cycles of the metals and limits potential food chain transfer to a restricted range of root consumer and decomposer organisms of *R. ulmifolius* (Marques et al. 2009). Despite the fact that the vegetation remains

abundant under a contamination environment, studies evaluating metal accumulation by *R. ulmifolius* are not yet common.

5.4.3 Transgenic Plants

Biotechnology has already been successfully employed to manipulate metal uptake and tolerance properties in selected plant species. Tolerance of transgenic plants to the presence of toxic levels of metals such as Cd (Kawashima et al. 2004), Zn, Cr, Cu, Pb (Bennet et al. 2003), As (Lee et al. 2003a, b), and Se (Berken et al. 2002) has been reported. A combination of transporter genes has also been used in rapidly growing plant species, leading to promising results (Lee et al. 2003a, b; Song et al. 2004). For example, in tobacco (*Nicotiana tabacum*) increased metal tolerance has been obtained by expressing the mammalian metallothionein, metal binding protein, genes. Possibly, the most spectacular application of biotechnology for environmental restoration has been the bioengineering of plants capable of volatilizing mercury from soil contaminated with methyl mercury. Methyl mercury, a strong neurotoxic agent, is biosynthesized in Hg-contaminated soils. To detoxify these substances, transgenic plants (*Arabidopsis thaliana* and *Nicotiana tabacum*) were engineered to express bacterial genes *mer*B and *mer*A. In these modified plants, *mer*B catalyses the protonolysis of the carbon–mercury bond with the generation of Hg^{2+}, a less mobile mercury species. Subsequently, MerA converts Hg (II) to Hg (0), a less toxic, volatile element, which is released into the atmosphere.

Overexpression of genes involved in phytochelatins (PCs) enabled the development of useful plants for phytoremediation, e.g. under Cd and As stress (Dhankher et al. 2002; Gasic and Korban 2007; Guo et al. 2008; Blum et al. 2010). Plants expressing *SRS1p*/*ArsC* and *Act1P*/*γ-ECS* showed two- to threefold elevated accumulation of As per gram of tissue in comparison to wild plants expressing *γ-ECS* or *Act1P* alone (Dhankher et al. 2002). Simultaneous overexpression of both *AsPCS1* and YCF1 in transgenic *Arabidopsis thaliana* resulted in longer roots and higher Cd and As accumulation than single-gene transgenic lines and wild plants (Guo et al. 2012). Transgenic *Brassica juncea*, grown either hydroponically or in soils, shows higher uptake of Se and enhanced Se tolerance compared to the wild species (Pilon-Smits et al. 1999). To engineer Se tolerance the seleno-cysteine methyltransferase (SMT) gene has been transferred from the Se hyperaccumulator *A. bisulcatus* to Se-non-tolerant *B. juncea*. SMT transgenic plants of *B. juncea* grown in a contaminated soil accumulate 60 % more Se than the wild type (Zhao and McGrath 2009 and literature reported therein) (Wenzel 2009; Rascioa and Navari-Izzo 2011).

5.5 Conclusion

Efficiency of plant growth depends on a lot of different factors and one of them that is able to significantly limit plant growth is the presence of heavy metals. The influence of heavy metals depends both on the kind of metals and their concentration in soil and also soil conditions (form of element presence in environment). Soil is an integral part of the environment and it influences plant development, independently of its contamination level. The presence of heavy metals and nutritional elements can significantly modify their accumulation (interaction), as well as stimulate/inhibit plant growth. For this reason, in the phytoremediation process a significant aspect is not only to determine the level of heavy metal contamination but also to analyse selected soil parameters, especially when we are interested in significant heavy metal accumulation connected with high biomass production. In our opinion, knowledge about polluted soil chemistry is especially important in the biomass aspect, which is one of the most interesting parts of studies in the 10 last years, as regards the increased energy requirement.

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Chapter 6
Metal Remediation via In Vitro Root Cultures

María del Socorro Santos-Díaz

6.1 Introduction

Some metals (Fe, Zn, Mn, Ni, Cu, and Mo) are essential for normal plant growth and development since they are nutrients and/or constituents of many enzymes and proteins. Nonessential heavy metals include As, Cd, Cr, Hg, Pb, Sb, and U. However, elevated concentration of metals can be detrimental to living organisms. They are toxic because they can replace metals in pigments or enzymes, disrupting the function of these molecules (Manios et al. 2003; Hou et al. 2007; Jayakumare et al. 2009). Heavy metals also cause oxidative stress, especially transition metals such as Fe and Cu. The toxicity of heavy metals is generally ascribed to their high affinity for nucleophilic groups. In fact, they are soft donors and will therefore readily bind to soft acceptors such as sulphydryl groups (Stohs and Bagchi 1995; Rivetta et al. 1997; Schutzendübel and Polle 2002).

Heavy metals are important environmental pollutants in soil, water, and air. Soil pollution differs from air and water pollution, because metals persist in the soil for a longer time than in other compartments of the biosphere (Lasat 2002). The main sources of contamination are agricultural fertilizers, pesticides, burning of fossil fuels, metalliferous mining, metallurgical industries, sewage sludge treatment, municipal wastes, and electronic industries (Wei and Zhou 2008; Wu et al. 2010). In addition to sites contaminated by human activity, other natural sources of heavy metal pollution include the mineral deposits in many regions of the planet, volcanic activity, and weathering of rocks (Carroll 1970; Hinkley et al. 2006).

There are around 430 plant species known, ranging from annual herbs to perennial shrubs and trees, that accumulate metals in large amounts. These species are of interest for potential use in phytoremediation of metal-contaminated environments

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(Suresh and Ravishankar 2004; Ghosh and Singh 2005; Padmavathiamma and Li 2007; Capuana 2011). Generally fast growing plants with high biomass and different kinds of root systems are used to clean up the pollutants. Metal removal can be done by taking up the metals into the roots or by transporting the metals from the roots up into the leaves (Pilon-Smits and Pilon 2002; Lasat 2002; Pilon-Smits 2005; Memon and Schröder 2009; Zhou et al. 2012).

6.2 General Characteristics of In Vitro Culture for Metal Removal

Another approach with great potential to study the phytoremediation process and the mechanisms involved in heavy metal tolerance is the use of in vitro cultures. Using this system, the analysis of metal removal can be performed under conditions that are more easily controlled than with soil-growing plants like medium composition, nutritional parameters, water potential, and plant growth regulator (PGR) concentration. In addition, the substance (organic and inorganic pollutants) and nutrient uptake is faster and more uniform because the barriers present in whole plant such as leaf wax, bark, cuticle, epidermis, and endodermis are not present in in vitro cultures (Buchanan et al. 2000; Hopkins and Hüner 2009).

In vitro culture involves growing plant cells and tissues on environmentally controlled conditions (temperature, photoperiod, and darkness), on defined medium, and in a microbe-free environment (George et al. 2008). Different media have been used for in vitro root culture, but the most employed are those derived from the original White medium (1934), Murashige and Skoog (MS) (Murashige and Skoog 1962), and B5 medium (Gamborg et al. 1968). In general, all media contain micro- and macronutrients, a carbon source (sucrose, glucose), vitamins, and PGR. The auxins are the primary PGR involved in the formation of adventitious roots and their promoting effect varied between species and cultivars (Blakesley et al. 1991; Nandagopal and Ranjitha Kumari 2007; Khalafalla et al. 2009). Besides auxins, the ability of a plant to produce in vitro roots depends on genotype, environmental conditions, and the level of nitrates in the culture medium (Kusakari et al. 2000; Sudha and Seeni 2001; Valim Reis et al. 2011). Liquid medium with or without agitation is more frequently used than solid media.

The in vitro systems allow the independent study of the complex interaction among plant/soil/microbiota, to evaluate the participation of specific enzymes, organic compounds, transporters, or peptides involved in the plant response to the pollutants (Boominathan and Doran 2002; Flocco and Giulietti 2007; Doran 2009). The axenic conditions in culture prevent microbial symbiosis disguising the metal uptake characteristics of plants grown in soil. Lynch (1982) reported that the soil associated with plant roots (rhizosphere soil) supports 10–100 times more microorganisms per gram than unplanted soil, due to a supply of carbon-containing compounds exuded by plant roots. Particularly, mycorrhizal fungal taxa, such as species like *Glomus, Gigaspora*, and *Entrophospora*, have been reported to be

associated with most of the plants growing in the heavy metal polluted habitats (Alves da Silva et al. 2005). The transport of the toxic metals absorbed by the mycorrhizal surface to the aerial part of the remediating plants is an obvious mechanism which can enhance the total uptake and transport of the toxic metals in a defined period, due to an increased surface area of the rhizosphere by the mycorrhizal associations (Khan et al. 2000; Schutzendübel and Polle 2002; Audet and Charest 2009).

An in vitro screening reduces not only the growth period and the treatment time length of the plants but also the space required for the experiments. Cell cultures are also a useful system for metabolic engineering and for obtaining rapid evidence of the ecotoxicological behavior of chemicals and heavy metals in plants with less analytical expense (Golan-Goldhirsh et al. 2004). Moreover, the environmental factor variability is also reduced, physiological activities can be increased by modifying the culture conditions (for example, employing biotic and abiotic stress), and it is easier to isolate and analyze metabolites (Shanks and Morgan 1999; Hu and Du 2006).

De-differentiated cells, such as callus or cellular suspension, and differentiated organs, such as roots and shoots, can be used for metal removal (Czuba 1987; Ros et al. 1992; Ramgareeb et al. 1999; Rout et al. 1999; Nehnevajova et al. 2007; Di Lonardo et al. 2011). When non-differentiated tissues are employed, genetic and epigenetic changes can be observed due to Somaclonal Variation (Lee and Phillips 1988). However, this variation and in vitro selection seem to be an appropriate technology for the development of new plant variants with enhanced metal accumulation and extraction properties (Jan et al. 1997; Herzig et al. 2003; Nehnevajova et al. 2007).

In vitro culture of roots and shoots allows indefinite propagation and experimentation using tissues derived from the same plant, avoiding the risks of variability between species (Pollard and Baker 1996; Huang and Cunningham 1996; Marmiroli 2007). This approach also allows the analysis of metal accumulation properties of each organ (Kartosentono et al. 2001; Nedelkoska and Doran 2000a) and the possibility to develop industrial bioreactor models (Kim et al. 2002; Giri and Narasu 2000). The in vitro root cultures are particularly important for studying the interaction of contaminants because they are in direct contact with pollutants, besides being metabolically very active. The roots not only participate in water and nutrient uptake but also synthesize and release several compounds. Root exudations include the release of ions, oxygen, and water but mainly consist of carbon-containing compounds from low- and high-molecular weight. Low-molecular-weight molecules include sugars and simple polysaccharides such as arabinose, fructose, glucose, maltose, and rammnose; amino acids such as arginine, asparagine, aspartic, cysteine, and glutamine; organic acids such as acetic, ascorbic, benzoic, folic, and malic acids; and phenolic compounds. High molecular weight compounds include flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes, and vitamins (Uren 2000; Bertin et al. 2003). Organ root culture is used for the study of the transport mechanisms of contaminants in roots with a focus on the interface among root tip,

root hairs, and the rhizosphere (Golan-Goldhirsh et al. 2004). The in vitro cultures can be established from small sections of roots bearing a primary or lateral root meristem. The culturing of adventitious root is an efficient means of biomass production because of fast growth rates and stability (Shanks and Morgan 1999).

6.3 Metal Removal by In Vitro Root Cultures

An efficient approach to establishing in vitro root cultures capable of tolerating metals is to obtain the tissue from plants growing in contaminated soils or water bodies. Using this protocol, in vitro root cultures from *Rubia tinctorum* and from the aquatic plants *Scirpus americanus* and *Typha latifolia* were established. These cultures require the addition of auxins to the culture medium to stimulate growth. Exposure of *R. tinctorium* cultures to Ag, As, Cd, Cu, Ca, Hg, In, Ni, Pb, Se, and Zn induced the formation of phytochelatins (PCs) and many of them also induced the corresponding desglycyl peptides (Maitani et al. 1996). The metal removal by *S. americanus* and *T. latifolia* root cultures were characterized by an initially rapid metal concentration decrease, followed by a slower decrease in the solution. The first stage could be related to a sorption process at the root surface, and the slower stage to an internalization of the metals. The cultures removed nearly 100 % of Pb^{2+} and Cr^{3+}, and 71–100 % of Mn^{2+} from culture medium after 6–8 day (Santos-Díaz et al. 2007). The quantification of metals in roots showed that Pb and Cr uptake was due to an absorption process while Mn was principally adsorbed to root surface. The concentration factors [CF - metal concentration in roots (mg g^{-1} dry weight)/metal concentration in medium (mg L^{-1})] calculated for *T. latifiolia* roots were 1,093, 1,473, and 122 for Pb, Cr, and Mn respectively, while for *S. americanus* roots were 2,198, 2,433, and 419 for Pb, Cr, and Mn, respectively. Thus, the root cultures of *S. americanus* were about two- to threefold more efficient at removing the metals than the *T. latifolia* roots (Santos-Díaz and Barrón-Cruz 2011).

6.4 Hairy Root Cultures for Phytoremediation Research

A variant from the in vitro root culture that has enormous potential is the hairy root culture, in which the root is infected with *Agrobacterium rhizogenes*, a Gram-negative bacterium that belongs to the *Rhizobiaceae* family. The hairy root phenotype also can be induced by mutation (Suzuki et al. 2008), but only the roots transformed with *A. rhizogenes* are considered in this chapter. This bacterium is attracted to wounded sites of the plant and subsequently induces the formation of adventitious roots in a wide range of plant species (Flocco and Giulietti 2007). During infection, *A. rhizogenes* transfers a segment of DNA from about 10–30 kb known as T-DNA, which is a portion of the large plasmid called the root-inducing plasmid (pRi) from about 200 kb (Giri and Narasu 2000). When T-DNA is integrated into the plant cell genome, it promotes the expression of enzymes that

direct the production of opines. These compounds are synthesized and excreted by the transformed cells and consumed by *Agrobacterium* as a nutrient source (Dessaux et al. 1992). According to the opines produced, the Ri plasmids can be classified into several lines: octopine, agropine, nopaline, mannopine, and the stereoisomers cucumopine/mikimopine (Hu and Du 2006; Veena and Taylor 2007). The T-DNA is defined by two border sequences from 25 bp in length and highly homologous in sequence. The T-DNA contains several genes, some of them involved in auxin biosynthesis and sensitivity (*aux)*, which cause differences in hairy root growth and morphology when compared to non-transformed roots (Meyer et al. 2000; Christey 2001; Chandra 2011). The genes *aux1 and aux2* seem to be responsible for auxin autotrophy of transformed roots. The genes *rol*, particularly *rol A-B-C-D* genes, affect the development of the infected plant cells, inducing the "hairy root" syndrome, the *rolB* gene being the most important. The product of *rolC* influences the metabolism of cytokinins and gibberellins (Estruch et al. 1991; Nilsson et al. 1993) while the rolB protein enhances auxin binding to the cell membrane (Filippini et al. 1994; Veena and Taylor 2007). For a more detailed description of the rhizogenic process, see Chandra (2011).

The extensive root proliferation induced by *A. rhizogenes*, generally considered an undesirable characteristic, may find good utility for phytoremediation due to the larger root surface to uptake the contaminant from the medium. Transformed root cultures present faster growth than non-transformed roots, are genetic and biochemical stable, and have hormonal autotrophy, that is, roots have the unique ability to grow in vitro without PGR and then are easily established and propagated in the laboratory (Shanks and Morgan 1999; Suza et al. 2008).

Root hair formation occurs within a specific region of the root, a short distance above the region of root elongation. Root hairs are short and develop on both primary and secondary roots. Interestingly, a root hair is a single cell that consists of a thin cell wall, a thin lining of cytoplasm that contains the nucleus, and a large vacuole containing cell sap (Gillaspy 2008). The hairy root disease is characterized by plagiotropic root growth, a high degree of lateral branching, and the profusion of root hairs, although the tissue maintains a highly differentiated and functional root organ. These roots also present a higher enzymatic degradation capacity due to the peroxidase, laccase, and oxygenase content (Flocco et al. 1998; Boominathan and Doran 2003b; Talano et al. 2003; Telke et al. 2011). The transformed roots offer the interesting property that the whole plant can be easily regenerated (Tepfer 1990; Giri and Narasu 2000). The hairy root cultures have proved to be successful in vitro systems for studying the phytoremediation process. Table 6.1 shows some examples of hairy root cultures used for metal removal.

6.5 Mechanisms of Tolerance to Heavy Metals Present on Hairy Root Cultures

The employment of hairy roots has been focussed on the extraction of Cd, Ni, U, Zn, and Cu. The mechanisms of tolerance of these metals are discussed below.

Table 6.1 Metal removal by hairy roots cultures

Metal pollutant	Species	Reference
Cadmium	*Calystegia sepium*	Metzger et al. (1992)
	Solanum nigrum	Macek et al. (1994)
	Thlaspi caerulescens	Nedelkoska and Doran (2000a)
	Adenophora lobophylla *Adenophora potaninii*	Wu et al. (2001)
	Thlaspi caerulescens *Nicotiana tabacum*	Boominathan and Doran (2003a, b)
Copper	*Hyptis capitata* *Nicotiana tabacum* *Polycarpaea longiflora* *Euphorbia hirta*	Nedelkoska and Doran (2000b)
Nickel	*Alyssum bertolonii* *Alyssum tenium* *Nicotiana tabacum*	Nedelkoska and Doran (2001), Boominathan and Doran (2002, 2003b)
Uranium	*Brassica juncea* *Chenopodium amaranticolor*	Eapen et al. (2003)
	Armoracia rusticana	Soudek et al. (2011)
	Daucus carota	Straczek et al. (2009)
Zinc	*Solanum niger*	Subroto et al. (2007)

6.5.1 Cadmium

Cd has been ranked as one of the major heavy metal hazards because it is mobile in soils, penetrates easily into the food chain, and presents adverse effects for human health (McLaughlin and Singh 1999). Among the first hairy roots tolerant to Cd are those obtained from *Calystegia sepium* (Metzger et al. 1992) and *Solanum nigrum* (Macek et al. 1994). These cultures accumulate about 1,100 μg g^{-1} and 24,455 μg g^{-1} (dry weight basis), respectively. However, hairy roots of *Thlaspi caerulescens* greatly surpass these concentrations, reaching 62,800 μg g^{-1} dry weight, accumulation which corresponds to 6.3 % dry weight. *T. caerulescens* roots localized the metal in the cell wall fraction during 7–10 days before allowing passage into the symplasm. This delay represents an important defensive strategy against Cd poisoning allowing time for activation of intracellular mechanism for heavy metal detoxification (Nedelkoska and Doran 2000a, 2003a). It has been found that *T. caerulescens* roots present high endogenous activities of catalase and superoxide dismutase, and high concentration of glutathione. In addition, the levels of H_2O_2 were maintained at nontoxic levels in the presence of metal. These results show that antioxidative defenses, specifically the induction of catalase activity, play an important role in the mechanism of tolerance to Cd in *T. caerulescens* roots (Boominathan and Doran 2003a). An increase in the activity of the enzymes superoxide dismutase (SOD) and peroxidase (POD) was also observed in *Cucumis sativus* L. hairy roots in the presence of low concentration of Cd (Zhang et al. 2009).

Interaction of Cd with organic acids in hairy root cultures has also been described, but plays a minor role. For example, the treatment with 20 μg g^{-1} Cd causes 13 % of the metal to be associated with organic acids in *T. caerulescens* roots (Boominathan and Doran 2003a). Distribution of Cd can be affected by treatment of roots with diethylstilbestrol (DES), an inhibitor of plasma membrane H^+ATPase that collapses the proton gradient. DES significantly altered the uptake and distribution of Cd in *T. caerulescens* cultures promoting a shift of Cd away from the cell walls into the symplasm, and a substantial increase in Cd concentration inside the cells. This result shows that Cd tolerance and hyperaccumulation are not affected by disruption of the transmembrane proton gradient (Boominathan and Doran 2003a). Other effects observed during Cd^{2+} exposure in hairy roots include changes in protein and PC content. Hairy roots from *Adenophora lobophylla* (species with low resistance to environmental stress) and *A. potaninii* (species widely distributed and vigorous growth) cultivated in 10–200 μM Cd increased 1.7- to 2-fold the protein content compared to control cultures. In addition, *A. lobophylla* accumulated more Cd, PCs, glutathione (GSH), and cysteine than *A. potaninii*. The shift of protein pattern and the lower accumulation of Cd in *A. potaninii* suggested a possible Cd exclusion system (Wu et al. 2001).

6.5.2 Nickel

Nickel occurs in soil with typical concentrations of 1–200 μg g^{-1}. It has phytotoxic effects on plant growth, photosynthesis, and membrane function (Pandolfini et al. 1992). Ni recovery from phytomining processes is important since the metal has relatively high commercial value (Robinson et al. 1997). To investigate Ni uptake, Nedelkoska and Doran (2001) compared Ni tolerance and Ni accumulation in hairy roots from *Alyssum bertolonii, A. tenium, and A. troodii*. In short-term experiments (9 h) the highest Ni content was 17,500 μg g^{-1} in *A. bertolonii,* and 1,100 μg g^{-1} in *A. tenium and A. troodii*. Growth of hairy roots from *A. bertolonii* was unaffected by the presence of 20 μg g^{-1} Ni, while growth of *A. tenium* was inhibited. The addition of EDTA to the medium improved growth of hairy roots and reduced the Ni content in root biomass, suggesting a protective effect of EDTA in chelating Ni ions in solution (Nedelkoska and Doran 2001). In hyperaccumulator plants, Ni is complexed with organic acids (malic, citric, malonic) or amino acids (histidine, glutamine). This mechanism of tolerance is also present in hairy roots but is not the main response. It has been reported that *A. bertolonii* hairy roots contained high constitutive levels of citric, malic, and malonic acids. After treatment with 25 μg g^{-1} Ni about 28 % of the total Ni was associated with organic acids and 85–95 % with the symplasm (Boominathan and Doran 2003a). In *A. bertolonii* hairy roots the Ni was distributed along length of roots including root tips (Boominathan and Doran 2003a), but virtually all metal is located in the symplasm.

After treatment of roots with DES a reduction on the entry of Ni into the symplasm was observed, reflecting that there is a coupling between the proton gradient generated by H^+ATPase and the transport of Ni (Boominathan and Doran 2003a).

On the other hand, *A. bertolonii* hairy roots presented 2.4–500 times higher levels of the antioxidant enzymes superoxide dismutase and catalase than *Nicotiana tabacum*. However, the exposure to Ni reduced the activity of these enzymes and increased the levels of H_2O_2 without affecting the growth of cultures. The authors suggest that other mechanisms for tolerating reactive oxygen species (ROS) must be involved, as enhanced vacuolar compartmentation (Boominathan and Doran 2002).

6.5.3 Uranium

Uranium presents both chemical and radiological hazards, the former being the greater risk factor (Ribera et al. 1996). Hairy root cultures have been used for several years to extract uranium from aqueous solutions. Metal removal was performed within a short period of incubation by hairy root cultures from *Brassica juncea* and *Chenopodium amaranticolor* (Eapen et al. 2003). At 500–5,000 μM uranyl acetate, a near-linear uptake was observed for *C. amaranticolor* roots, whereas *B. juncea* cultures showed saturation. For all concentrations used, 90 % of the uranium was taken up by the root tissue within 10 h of treatment, and both cultures were able to accumulate 8,000 μg g^{-1} dry weight. Hairy roots from carrot *and Armoracia rusticana* also are able to remove uranium from medium (Soudek et al. 2011; Straczek et al. 2009). Accumulation of uranium in *A. rusticana* was very fast, reaching the maximum at 1–2 h, and was dependent on initial concentration (50–500 μM), indicating that metal uptake is due to a simple or facilitated passive diffusion more than an active transport. Roots of carrot were more sensitive to uranium concentration presenting toxicity symptoms at 6 days at 15, 20, and 30 mg L^{-1} uranium (Straczek et al. 2009). Accumulation of uranium is influenced by the phosphate concentration on culture medium. On *C. amaranticolor* and *B. juncea* root cultures the phosphate reduces uranium uptake (Eapen et al. 2003); meanwhile in *A. rusticana* cultures, this ion improved 50 % metal accumulation (Soudek et al. 2011). It has been described that in a pH range from 4 to 7.5, uranium exists as a phosphate complex which can be transported to the aerial parts (Vandenhove et al. 2007). Considering that the pH of culture media usually is 5.5–5.7, the formation of uranium–PO_4 complexes under in vitro conditions is possible. Then the different responses observed in *A. rusticana* vs *C. amaranticolor* and *B. juncea* cultures relative to phosphate content could be related to their capacity for internalizing the uranium–PO_4 complex. *A. rusticana* root cultures presented a light increase in peroxidase and glutathione-S-transferase activities in the presence of uranium but data are not conclusive. Further work is needed to explain the mechanism of tolerance in *A. rusticana* root cultures.

6.5.4 Zinc and Copper

The annual worldwide release of Zn to environment exceeded the levels of other heavy metals reaching 1,350,000 Mg (Singh et al. 2003). Zinc is also a dominant heavy metal that pollutes rivers in several countries (Mason 1991; Pistelok and Galas 1999; Jain 2004). *Solanum niger* is a hyperaccumulator of zinc and therefore is a good candidate to establish in vitro root cultures. Subroto et al. (2007) studied the ability of the hairy root cultures of this species to absorb zinc. Two strains of *S. nigrum* hairy roots were isolated, strain A4 and strain K1 (control). Both strains are able to grow in medium supplemented with 13.98 mg L^{-1} Zn. The strain A4 was capable of accumulating Zn from the culture medium better than the strain K1. However, the two strains of hairy roots presented similar patterns of growth and metal absorption and were able to remove as much as 98 % of the Zn from the culture medium within 18 days. Both strains A4 and K1 actually reached maximum Zn accumulation at day 9. Strain A4 accumulated slightly more Zn than the strain K1. Only small amounts of Zn underwent an uptake–release pattern, suggesting that metal strongly binds to cellular sites.

Regarding copper, its high toxicity to plants is due to inhibition of the activity of many enzymes, photosynthesis, pigment synthesis, alteration of membrane integrity, and blocking of photosynthetic electron transport, leading to the production of free radicals (Fernandes and Henriques 1991).

Genetically transformed hairy root cultures were established from *Hyptis capitata*, which is a widespread weedy species; *Polycarpaea longiflora*, belonging to the *Caryophyllaceae* which includes copper indicator species; *Euphorbia hirta*, a rhizomatous herb; and *N. tabacum*. These cultures were screened for their capabilities to uptake copper. After a short-term exposure to 1,000 μg g^{-1} Cu, the *H. capitata, P. longiflora*, and *N. tabacum* hairy root cultures accumulated similar copper levels, but the Cu content in *E. hirta* hairy roots was 28 % lower. Equilibrium Cu levels for the four species represent average concentration factors of 3.8–5.6 relative to the Cu initial concentration provided. Thus, the establishment of hairy root cultures from a range of plant species demonstrates the utility of this system for screening plants with capabilities to uptake metals (Nedelkoska and Doran 2000b).

6.6 Scaling Up of Hairy Roots and Bioreactors

Advances in design of proper bioreactors for hairy roots growth are being of great interest, since scale-up will allow the integration of this technology to industrial processes. Several challenges must be surpassed for commercial exploitation. For example, mechanical agitation causes wounding of hairy roots and leads to callus formation; meristem-dependent growth of root cultures in liquid medium results in a root ball with young growing roots on the periphery and a core of older tissue inside, and it is difficult to have a good distribution of roots which affects the supply of nutrients and oxygen (Kim et al. 2002). However, techniques of inoculum handling, root homogenization (Ramakrishnan et al. 1994), and an inoculation

apparatus (Kawamura et al. 1996) have been developed to solve some of these problems. In addition, mist, trickle bed, and hybrid reactors have been proved to be very effective for growing hairy roots. On mist reactors, nutrients and water are sprayed over the surface of the roots. Using ultrasonic transducers, the droplet sizes are usually micron scale (0.5–30 μm). In gas-phase reactors, nutrients are usually delivered as droplets and the roots are exposed to air or other gas mixtures virtually eliminating oxygen deficiency in dense root beds (Kim et al. 2002). In addition, bioreactors (10,000–20,000 L) operated with bubble columns have been developed for *Panax ginseng* hairy root proliferation (Sivakumar et al. 2006; Choi et al. 2006), showing that the technology to obtain a huge mass of roots is now available. The next challenge will be to apply this methodology for remediation studies.

6.7 Conclusions and Future Directions

Hairy roots can be generated from many plant species by infecting them with *A. rhizogenes*. The versatility of hairy roots makes this system very attractive to study diverse physiological aspects of plants and to improve the efficiency of phytoremediation due to their high proliferative capacity. Hairy roots are also a very interesting model for molecular genetic studies of metal accumulation. The use of microarrays, expressed sequence tag (EST), and quantitative trait loci (QTLs) could be invaluable tools to identify specific genes involved in metal tolerance. In addition, genes can be isolated from various organisms, including bacteria, fungi, plants, and animals, and introduced into hairy roots for testing the efficacy of transgenes and the enzymes they encode for the removal of hazardous environmental pollutants. Transgenic plants regenerated from in vitro root cultures would be more efficient to remove metals. Aquatic plants regenerated from hairy roots would be another approach to clean up water bodies that are highly contaminated.

Previous reports (Doran 2009) have described the lack of suitability of direct phytoremediation applications using in vitro cultures due to several important restrictions. These include the requirement of sterile conditions for the proper development of roots, the heterotrophy of cultures which require sugars to be provided in medium, the enormous mass required for the treatment of environmental wastes, and the cost of production of this biomass. However, bioreactors could be used for the remediation of moderate or small water volumes, as industrial effluents, which usually are poor in organic matter. Substitution of culture medium by a nutritive solution with the minimum concentration of mineral salts would restrict the microbial proliferation and would support the maintenance of cultures. In our laboratory we observed practically the same growth rate of *S. americanus* root cultures on commercial hydroponic solution without sucrose compared with MS medium with sucrose (data not published). Development of *S. americanus* cultures obviously required the addition of PGR to stimulate growth, but hairy root cultures normally do not need them; therefore they can be propagated easily.

Another alternative would be the use of a two-step process. The first step would focus on building up the biomass on big bioreactors, and the second one on the metal removal. This strategy has been successfully used for secondary metabolite production (Bourgaud et al. 2001).

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Chapter 7
Use of Wetland Plants in Bioaccumulation of Heavy Metals

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7.1 Environmental Contamination

Environmental pollutants due to dispersal of industrial and urban wastes generated through anthropogenic activities have become a major global concern. Most of the pollutants once enter into the environment get accumulated in soils and aquatic environments, creating wide spread contamination that vary in composition and in concentration. Several factors are responsible for the migration of contaminants like controlled and uncontrolled disposal of organic and inorganic wastes, accidental and process spillages, inadequate residue disposal, mining, and smelting of metalliferous ores, sewage sludge application to agricultural soils, etc. (Ghosh and Singh 2005; Kavamura and Esposito 2010). Steady deterioration of the environment due to pollution and its ailing effects to mankind is among the major concerns worldwide.

Heavy metals (elements with metallic properties like ductility, conductivity, stability as cations, ligand specificity, etc., with an atomic number >20 and having specific weight >5 g cm^{-3}) constitute an exceptionally diverse assembly of elements largely diverse in their chemical characteristics and biological functions. Though most of the metals are essential, all are toxic to organisms at higher concentrations due to production of free radicals that cause oxidative stress or

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replacement of essential metals in pigments or enzymes disrupting their function (Prasad and Freitas 2003). Thus higher proportion of heavy metal contamination destroys the biodiversity by making the area inappropriate for propagation of life forms. A number of these metals, due to their toxicity, are found in the top 20 on the 2007 CERCLA Priority List of Hazardous Substances, including arsenic (ranked first), lead (ranked second), mercury (ranked third), cadmium (ranked seventh), and chromium (ranked 17th) (CERCLA 2007).

7.2 Heavy Metals as Contaminants of Environment and Its Effects

Both natural and anthropogenic sources are responsible for release of heavy metals into the environment. Dumping of untreated industrial wastes and different metal mining operations are the major concern of heavy metal pollution (Hutton and Symon 1986; Nriagu 1989). Even long after the dumping activities have ceased, the released metals continue to persist in the environment and gradually contaminating all the downstream water bodies, like rivers and streams or run-off to the sea (Nriagu 1989). The metals may then be accumulated in sediments of water bodies or seep into the underground water thereby contaminating the underground water resources.

Widespread heavy metal pollution due to industrial activities has been reported from different parts of the world. An estimated 52 million hectares in the EU alone—more than 16 % of the total land area—are affected by some level of soil degradation (Peuke and Rennenberg 2005). Reports suggest that many countries like Japan, Indonesia, China (with Cd, Cu and Zn), Greece (Cu and Pb), and Australia (Cu, Pb, Cu, Ni, Zn, and Cd) are contaminated with heavy metals (Herawati et al. 2000; Zantopoulos et al. 1999). In India, according to the published information, several places are contaminated with metals due to industrial activities causing a major environmental problem. States in India with major industries like Gujarat, Maharashtra, and Andhra Pradesh have been reported to add almost 80 % of hazardous waste (including heavy metals) in India (INSA, A Position Paper 2011). In general, heavy metal toxicity can cause chronic degenerative diseases with symptoms like, pain in muscle and joints, gastro-intestinal disorders, vision problems, chronic fatigue, susceptibility to fungal infections, mental disorders, genotoxicity, and cancers (Shanker et al. 2005; Nath et al. 2005). Industrial workers, malnourished people, and pregnant women are vulnerable to the toxicity of the heavy metals. Crippling effects of fluoride and arsenic toxicity due to nonavailability of safe water for drinking and farming has now become a major public health problem.

Our present understanding suggests that heavy metals like arsenic (As), lead (Pb), mercury (Hg), and cadmium (Cd) do not possess any significant biological functions (Nriagu and Pacyna 1988; Duruibe et al. 2007; Chetia et al. 2011). As for example, in the environment, Pb is known to be toxic to plants, animals, and

microorganisms. Further, Pb influences child's s nervous system, slowing down nerve responses affecting learning abilities and behavior. Again, Hg when released into the environment, is retained in the soil in the form of complexes of toxic ionic mercury (Hg^{2+}), which may subsequently be converted into methylmercury and is likely to be accumulated within different organisms (Ke et al. 2001; Brim et al. 2000). Methylmercury poisoning mainly affects the brain (similar to lead) of children, even causes detrimental consequences at embryonic stages entering through placenta. While, Cd is toxic and mostly affects kidneys, resulting in kidney dysfunction and increased excretion of proteins in urine (proteinuria). However, Cr differs from Cd, Pb, and Hg by being essential in form of Cr(III) to humans and animals. Widespread effect is caused by exposure to chromium (especially Cr(VI) compounds) which are generally considered the most toxic (assumed to cause cancer) (Shanker et al. 2005).

7.3 Heavy Metal Remediation and Ecosystem Restoration

Heavy metals are natural trace components of the aquatic environment, but background levels in the environment have increased especially in areas where industrial, agricultural, and mining activities are widespread (Bryan and Langston 1992). Heavy metals released into the environment from different sources as direct input or surface runoffs find their way into aquatic systems and consequently, aquatic organisms may be exposed to elevated levels of heavy metals (Kalay and Canli 2000). Heavy metals may affect organisms directly by accumulating in their body or indirectly by transferring to the next trophic level of the food chain.

Schaller et al. (2011) reported that by the end of the 1980s, only mining activities damaged approximately 9,300 km of streams and rivers and 72,000 ha of lakes and wetlands worldwide. Different water bodies worldwide receive more than 180 million tonnes of perilous mine wastes (more than 1.5 times of all the municipal waste dumped in US landfills in 2009), discarded by the mining companies every year (Earthworks and mining watch 2012; USEPA 2009a). This poses serious threat of heavy metal and different chemical contamination of vital water bodies. Usually metals in soil may be linked with different fractions. It may be as free metal ions (e.g., Cd^{2+}, Zn^{2+}, Cr^{3+}) and soluble metal complexes, adsorbed form to other inorganic soil constituents, complexes with soil organic matter ($CdCl_3^-$, $CdSO_4^0$, $ZnCl^+$ etc.), associated as a structure of silicate minerals, linked with mobile organic or inorganic colloidal substances or precipitated such as oxides, hydroxides, and carbonates (Tessier et al. 1979; Lasat 2000). Therefore, the concentration of a metal in the solution of soil or sediment is the sum totals of various fractions like concentrations of free ion of the metal plus soluble organic and inorganic metal complexes plus the metals associated with movable materials. Inorganic and organic ligands are the main components of soluble metals complexes. Inorganic ligands (e.g. SO_4^{2-}, Cl^-, OH^-, PO_4^{3-},NO_3^-, and CO_3^{2-}) and their metal complexes in soil are well characterized. Soil organic ligands vary

widely, ranging from low-molecular weight aliphatic, aromatic, amino acids, and fulvic acids (soluble portion) and thus organic complexes with metals is poorly defined (McLean and Bledsoe 1992). The transport of metals in the soil solution is considerably affected by the complexes formed with the soil matrix. Binding of metals with organic matters like plant exudates or humus occupies a continuum of reactive sites, ranging from weak to strong chemical bonds.

Traditional methods of mitigating metal contamination in soils and water include various isolation, extraction, immobilization, and toxicity reduction methods, including isolation or physical barrier (i.e., concrete, steel); chemical solidification or stabilization; hydrocyclone, fluidized bed, or flotation processes; electro kinetic processes; soil washing; and pump-and-treat systems (Mulligan et al. 2001). These methods for metal sequestration are prohibitively expensive (around $400 to $750 billion in the USA alone), energy intensive, and can reduce the fertility and bioactivity of soils. The tremendous economic costs of technology-based environmental remediation are not a viable option for most of the developing countries to go for such expensive outlay (Mulligan et al. 2001). Moreover, there is no effective way to deactivate radioactive materials, except to allow them to decay in a site. Unfortunately, many of radionuclides have very long half-lives (e.g., Sr-90: 28 years; Cs-137: 30 years; Pu-239: 24,100 years; Tc-97: 2.6 million years; and U-235: 7.13 million years). Further methods like incineration and land-filling also raise several questions like, air/soil/groundwater pollution, and translocation of contaminants from one site to another. The problem of heavy metal contamination persists even with the disposal of incineration residues like land filling. Though the rate of heavy metals mobility in landfills is very low, however, landfills are not the permanent solution to contain heavy metals for long times. The high cost and other limitations of technology-based remediation is perhaps the driving factor in the development of alternative remediation technologies (Korda et al. 1997; Brim et al. 2000).

Natural biodegradation can reduce waste and help in cleaning up of varied types of environmental contaminants. By definition, bioremediation (includes phytoremediation) is the use of living organisms (bacteria and fungi or plants) for degrading or detoxifying the hazardous environmental pollutants into less toxic forms (Robles-González et al. 2008; Cozzarelli et al. 2010). Specific contaminants may be targeted for bioremediation like degradation of chlorinated hydrocarbons or such other compounds by indigenous or exogenous bacteria. Nevertheless, biodegradation is a complex process involving orchestrated actions of a string of organisms (Cho et al. 2000).

Microorganisms have the capacity to remove many contaminants from the environment by a diversity of enzymatic process. Oxidation of toxic, organic components to non-toxic product is one of the common types of bioremediation process taking place by microorganism having wide phylogenetic diversity. Aromatic hydrocarbons, xenobiotics and pesticides, and range of organic contaminants (Landmeyer et al. 2010; Landmeyer 2011) are usually aerobically degraded, as oxygen is the most commonly preferred electron acceptor in microbial respiration. However, a number of microorganisms along with plants (phytoremediation), as a result of their versatility, adaptability, and diversity in the environment,

are considered to be the best candidates among all living organisms to remediate most of the environmental contaminants, especially inorganic contaminants like heavy metals into the natural biogeochemical cycle (Lovley 2003).

7.4 Phytoremediation: The Process Overview

Phytoremediation (Ancient Greek: phyto-"plant", and Latin remedium-"restoring balance") is a low-cost, natural solar-powered, environment-friendly, less/no maintenance, aesthetically pleasing technology that can treat diverse environmental pollutants including heavy metals. It is a better alternative to costly mechanized methods like extraction, pump and treat systems, or soil washing.

Phytoextraction, phytostabilization, rhizofiltration, and phytovolatilization are the basic mechanisms of phytoremediation technology by which plant uptake heavy metals. Phytoextraction involves the uptake/absorption and translocation of heavy metals by roots into the above ground parts (shoots) of the plants. Shoot part of the plant may be harvested periodically and incinerated for energy and the ash may be recycled for metals. In general, metal uptake and phytoextraction coefficients decrease in the order $Cr^{6+} > Cd^{2+} > Ni^{2+} > Zn^{2+} > Cu^{2+} > Pb^{2+} > Cr^{3+}$ (USEPA 2000). Immobilization of contaminants using certain plant species in the soil and groundwater is the basis of phytostabilization. The process involves either adsorption of contaminants onto roots or precipitation within the root zone avoiding their exodus in soil or movement by erosion. Rhizofiltration is the technology for cleaning up communal wastewater, where adsorption or precipitation onto plant roots or absorption and sequestration of contaminants take place in the roots that are present in the adjacent solution (Fig. 7.1). Contaminant uptake and transpiration by a plant is known as phytovolatilization. It occurs as along with the growth of the plant as it takes up water along with the pollutant (i.e., for Hg, Se, As) (Noctor et al. 1998; Meagher 2000; Lasat 2000; Salido et al. 2003; Ghosh and Singh 2005; Tangahu et al. 2011; Using phytoremediation to Clean Up Sites http://www.epa.gov/superfund/accomp/news/phyto.htm; accessed on 30-8-2012). Again, many plants have the capacity to accumulate heavy metals at much higher concentration without affecting their metabolic process. A plant of this category may be hyperaccumulator when it can concentrate the pollutants in a least proportion which differs according to the pollutant concerned (e.g., more than 1,000 mg kg^{-1} of dry weight for chromium, copper, cobalt, nickel, or lead or more than 10,000 mg kg^{-1} for zinc or manganese) due to adaptive evolution towards hypertolerance or phytotolerance. Metal hyperaccumulation in plants may lead to several interactions like defense, mutualism (mycorrhizae, pollen, and seed dispersal), interferences with neighboring plant species, commensalism, and biofilm formation (Baker and Brooks 1989; Barron 2003; Michel et al. 2007; Burken et al. 2011).

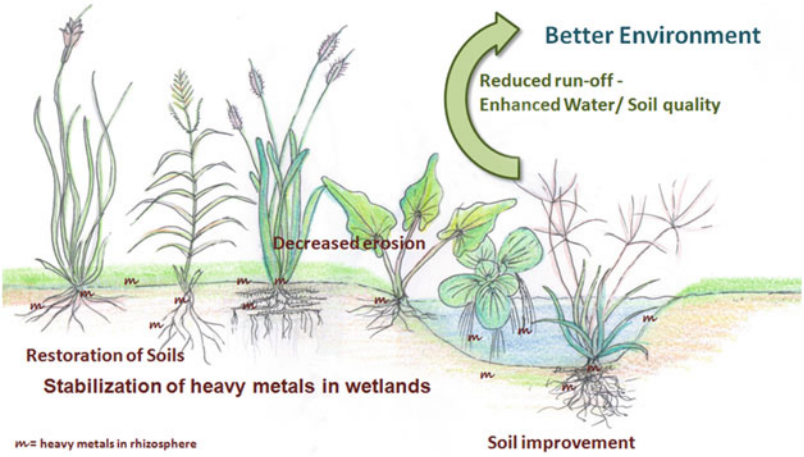

Fig. 7.1 Schematic representation of stabilization of heavy metals by wetland plants

7.5 Wetland Ecosystems: Introduction and Importance

Wetlands are areas where water is the primary factor controlling the environment and the associated life forms. In these regions, saturation with water determines the soil development character and the growth of flora and fauna living in the area. These are transitional zones that occupy an in-between position flanked by dry land and open water. Thus, wetlands may support both aquatic and terrestrial species as well as acting as "Kidney of Nature" (USEPA 1995). In broader sense, wetlands are "areas of marsh, fen, peat-land or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed 6 m. Wetlands may also incorporate riparian and coastal zones adjacent to the wetlands, and islands or bodies of marine water deeper than six meters at low tide lying within the wetlands" (Article 1.1 and 2.1 of Ramsar Convention on wetlands). Found all over the world except Antarctica, wetlands vary widely because of diversity in topography, climate, soils, hydrology and water chemistry, vegetation, and other factors, including human interference. It has been estimated that about 570 million hectares (5.7 million km^2)—roughly 6 % of the Earth's land surface is wetland. Among this, 2 % are lakes, 15 % floodplains, 20 % swamps, 26 % fens, and 30 % bogs (Mitsch and Gosselink 2000). Wetlands are some of the most productive and dynamic habitats in the world. Long regarded as wastelands, wetlands are now regarded as vital component in the landscape that affords plentiful of valuable services for the environment, wildlife, and people (USEPA 2001). Wetlands can be regarded as biological supermarkets due to species richness and diversity and their functions are basically the interrelations between different physicochemical and biological entities present in the area. These functions include flood control, groundwater replenishment, nutrient retention and export, water purification,

shoreline stabilization and storm protection, sediment and nutrient retention, climate change mitigation, reservoirs of biodiversity, wetland products, recreation tourism, and cultural value (Reed 1991). For conservation and wise use of natural resources of wetlands, the "Convention on Wetlands (Ramsar, Iran, 1971)" is an intergovernmental treaty adopted on 2 Feb 1971 in the Iranian city of Ramsar, which is now popularly known as "Ramsar Convention." More than 1,631 wetlands have been designated for inclusion in the List of Wetlands of International Importance, covering around 193,553,062 hectares, involving 162 contracting parties (source: http://www.ramsar.org, accessed on 30 Aug 2012).

Services provided by the wetland ecosystems are hugely valuable to people worldwide. It is not possible to measure tangible values of the diverse services towards the Nature by wetlands. A lot of these services like water purification, groundwater recharge, or values related to aesthetic and cultural are not instantly apparent to a wetland. However, the value of these wetlands and their associated ecosystem services has approximately been estimated at US$14 trillion annually (Millennium Ecosystem Assessment 2005). Role of wetland biota and their significant abilities in removal and/or breakdown of pollutants, xenobiotics, nutrients and other compounds through detoxification, retention, recovery, and removal help a lot for the pollution control strategies throughout the world. However, water or soil quality of the region influences the maximum amount of waste that can be recycled or immobilized on a sustainable basis (de Groot et al. 2006).

7.6 Restoration of Metal-Contaminated Wetland by Phytoremediation

Throughout the world, water quality of the wetlands is severely affected by increasing anthropogenic and industrial activities and untreated waste dumping. Thus, wetland pollution and degradation is increasingly becoming a major issue reflecting changes in water quality, quantity, flow rates, and in species composition. Filling up or drainage or diversion of wetlands for development, farming, and mosquito control also cause degradation of wetlands. Further, diverse organic and inorganic contaminants like heavy metals pollution above a limit gradually annihilate these important regions.

Runoffs (may be storm water or nonpoint source pollution) usually carry diverse metals into the wetlands. Wetlands have the potentiality to improve naturally the water quality, and therefore, wetlands may be used to treat runoff of different contaminants. Wetlands do provide valuable water quality protection for downstream rivers, lakes, and estuaries (USEPA 2009b) that signify their importance to protect and restore such wetland areas. To replicate the functions of filtering pollutants of wetlands, artificial wetlands like marsh or swamp may be created to restore habitat. The constructed wetlands are usually constructed in such a way to involve similar wetland vegetation's, soils, and their associated microbial assemblages like that of

natural systems to improve water quality of wastewater discharge or sewage treatment, storm water runoff, land reclamation after mining or refineries (USEPA 2004).

The uptake and accumulation of different elements in the wetlands mostly depends upon the diverse factors like metal concentrations in soils, organic matter content, pH, cation exchange capacity, and diversity of macrophytes present in the region. However, it is well proven that the concentration of metals in soil is the predominant factor; additionally, soil pH also governs the uptake of metal by plants (Jung 2008). Further, active uptake of elements through their "nutrient pumps" (Odum 1988), promote immobilization of metals in high concentration in plant tissues endorsing the use of such wetland plants in phytoremediation for both natural and constructed wetlands for wastewater treatment (Kadlec and Knight 1996).

Among the varied phytoremediation technologies, in wetlands, plants may be used either for immobilization and storage of metals (phytostabilization) below ground in roots and/or soil, or "phytoextraction" in which hyperaccumulators may be used to remove metals from the soil and concentrate them in aboveground tissues (McGrath and Zhao 2003). The process of phytoextraction mostly needs maintenance as accumulator plants must be, in turn, harvested and disposed of to prevent recycling of accumulated metals when the plants decompose. Again, the mechanical aspects of harvesting plants would be disparaging to wetlands comprised of rooted plants. However, for application, the patterns and processes of metal uptake, distribution, and removal by different species of wetland plants needs to be taken care for. This data is very important to monitor the effects on the residence time of metals in plants and in wetlands, and the potential release of metals into the system (may be dead plant tissues); otherwise, wetlands themselves in due course would turn out to be the source of metal contamination to the vicinity. Thus, highly metal enriched deceased plant material is a concern as the elements may be released again into the surrounding, polluting through leaching and mineralization by litter adsorption or microbial immobilization. Accordingly, application of wetland plants for wastewater treatment should be done after proper scientific study as limitation of the plants to sequester the contamination and the assimilative capacity should also be taken care for (Verkleij and Schat 1990).

7.7 Wetland Sediment and Contaminant Uptake by Plants

Generally, sediments are the sites of sink for metals. However, quality of soils also differ in terrestrial and wetland systems. In terrestrial systems, soils are mostly oxidized, but in wetlands, due to saturation of water, sediments become anoxic in nature. Thus the bioavailability of the metals is low in wetland areas. Further, metal bioavailability also depends upon metals association with different fractions. Most available form is water-soluble fraction of metals. Metals associated with inorganic compound or humus materials or adsorbed to hydrous oxides are less available than dissolved in aqueous solution. However, metals are essentially unavailable when

they are precipitated as insoluble forms or bound within the crystalline mineral lattice (Gambrell 1994). Therefore, the uptake of contaminants and rhizosphere actions depend upon the state of metals and also the capability of the particular plant and its root characteristics (Gleba et al. 1999; Salt et al. 1998; Williams 2002; Prasad 2004). Several researchers have explored the contaminant uptake and its method by plants which can help to optimize the factors to improve the performance of plant uptake. In a polluted environment, plants may act both as "accumulators" and "excluders". Accumulators survive despite concentrating contaminants (biodegrade or biotransform into inert forms) in their aerial tissues. The excluders restrict contaminant uptake into their biomass (Tangahu et al. 2011). However, for both types of the plants, the common mechanisms involved in the uptake, translocation, and storage of toxic elements are aided by plant-produced chelating agents and plant induced pH changes and redox reactions. The range of known transport mechanisms or specialized proteins embedded in the plant cell plasma membrane involved in ion uptake and translocation include proton pumps (-ATPases that consume energy and generate electrochemical gradients), co- and anti-transporters (proteins that use the electrochemical gradients generated by -ATPases to drive the active uptake of ions), and channels (proteins that facilitate the transport of ions into the cell). Each transport mechanism is likely to take up a range of ions (Tangahu et al. 2011). However, after uptake, transportation of metal ions to the shoots is desirable, which will help to harvest the plant biomass (Salido et al. 2003). Avoiding metal toxicity is the interesting property of the metal-accumulators having higher concentration stored within the body. Multiple mechanisms are involved for this purpose like storage contaminants in the vacuole or the process of evapotranspiration that helps in moving contaminants into the plant shoots. Translocation and accumulation of contaminants in plant shoots are desirable as shoots can be harvested from time to time, while leaving the original soil undisturbed. Usually hyperaccumulators thrive in the metal infested wetlands, require little maintenance and produce high biomass, although few plants perfectly fulfill these requirements (Salido et al. 2003). It has been reported by Tangahu and his coworkers that hyperaccumulator plant species can concentrate heavy metals like Cd, Zn, Co, Mn, Ni, and Pb up to 100 or 1,000 times those taken up by nonaccumulator (excluder) plants (Tangahu et al. 2011). However, to mobilize metal ions and increase the bioavailable fractions to plants, microorganisms like bacteria and fungi, living closely associated in the rhizosphere significantly contribute towards this action (Tangahu et al. 2011).

7.8 Role of Rhizobium of Wetland Plants

As mentioned elsewhere, wetlands mostly contain anoxic sediments. Root zone of many of the wetland plants have the capability to mobilize and uptake metals from the anoxic area by either oxidizing the sediments through the movement of oxygen downwards through aerenchyma tissue (Moorhead and Reddy 1988) or by

acidification of the rhizosphere by plant exudates (Doyle and Otte 1997). The oxidation usually remobilizes the metal contaminants in the exchangeable form (*Avicennia* species of mangroves) in wetland sediments (de Lacerda et al. 1993). However, in the case of the plant *Typha latifolia* it is reported that, after oxidizing the rhizosphere zone, decreased the pH within 1 cm of the roots and increased the concentration of soluble zinc in and around the roots (Wright and Otte 1999). Changes in sediment Eh and pH can cause changes in metal speciation, solubility, and flux. With an increase in redox potential and pH, Pb uptake into roots and shoots of rice plants (*Oryza sativa*) decreased, while Cd uptake increased with a decrease in pH and an increase in redox potential (Reddy and Patrick 1977). Under dry (more oxidized) soil condition better availability and uptake of Cd was seen in a number of wetland plant species (Gambrell 1994). The wetland plants having larger and elaborated root system may indicate better efficiency to oxidize and mobilize metals of anoxic sediments at rhizosphere level (Ravit et al. 2003).

Mobilization and rates of uptake of metal by plants also depends upon the different forms ("species") of the same metal. Diverse group of bacteria present in the sediments of marsh lands and associated with plant roots have the capacity to reduce the very toxic form of metals to less toxic one. As for example, reduction of highly toxic Cr(VI) to the less toxic form, Cr(III) (Pardue and Patrick 1995), methylate arsenic into volatile (e.g., methylarsines) or nonvolatile (e.g., methylarsonic acid and dimethylarsinic acid [DMAA]) (Bentley and Chasteen 2002), help the plant to mobilize the same within their tissue system. Few aquatic plants like *Ceratophyllum demersum* and *Elatine triandra* are reported to synthesize lipid-soluble arsenic compounds to alleviate the toxicity of the arsenic (Tamaki and Frankenberger 1992; Zheng et al. 2003). Roots were found to be the major site of accumulation for inorganic arsenicals, while DMAA was readily translocated to the shoots (Carbonell-Barrachina et al. 1998). It has been observed by several workers that roots of several wetland plants carry metal-rich (5–10 times more than surrounding sediments) rhizoconcretions or plaques composed mostly of iron hydroxides and other metals like manganese that are mobilized and precipitated on the root surface. These plaques are thought to act like a barrier for some metals but cooperative for few others (Mendelssohn and Postek 1982; Vale et al. 1990; Sundby et al. 1998; Ye et al. 1998; Weis and Weis 2004).

7.9 Role of Microbial Association/Symbiosis with Plant Root

Microbial association and symbiosis at the root zone or rhizosphere of the wetland plants play an important role in the accumulation of metals. Many interesting studies have been done in this aspect. It was reported that, when rhizosphere bacteria were inhibited with antibiotics, plants accumulated lower concentration of metals; on the contrary when grown axenically with added bacteria, accumulated more of these metals than axenic controls (de Souza et al., 1999; Stout et al., 2010). Plants like *Scirpus robustus* and *Polypogon monspeliensis* were found to accumulate lower

concentrations of Se and Hg when they were treated with antibiotics than their normal counterparts (de Souza et al. 1999). Similarly, mycorrhizae (symbiotic fungi associated with roots), by increasing the absorptive surface area of root hairs, assist plant either assimilating metals (Meharg and Cairney 2000) or protect plants by restricting the uptake of metals by immobilizing them (Khan et al. 2000). Thus periphyton sometimes associated with freshwater wetland plants (as for example, *Phragmites australis*) help in enhancement and the ability to accumulate and retain metals (Lakatos et al. 1999).

Microbial community plays a major role in phytoremediation of wetland plants. Community diversity and structure of microorganisms, their enzymatic activity, and microbial-mediated edaphic processes (C and N mineralization, decomposition) mostly depend upon metal(s) concentration(s) of the root zone of wetland plants (Baath 1989; Roane and Kellogg 1996; Bruins et al. 2000) that also help plants to develop mechanisms to ameliorate toxicity of metals and to tolerate and/or resist multiple metal sequestration in a complex polluted environment (Nies 1995, 1999; Giller et al. 1998; Bruins et al. 2000; Pal et al. 2004). However, metal concentration plays a critical role in alteration in species composition, density, and biomass reduction of microorganisms (Baath 1989; Chander and Brookes 1993; Chander et al. 2001; Baath et al. 2005). It is reported that metals like Cd, Cr, Mo, Ni, Pb, and Zn shift the bacterial community with increase in the diversity of Gram positive bacteria with members from Proteobacteria, Acidobacteria, Verrucomicrobia, and Chlorobi groups in serpentine soils (Mengoni et al. 2004; Akerblom et al. 2007). However, few bacterial groups remain unchanged to certain metals with higher concentrations. As for example, actinobacterial community diversity remained unaffected with additional inputs of Pb and Zn in a Pb/Zn-contaminated grassland soil, though community diversity became reduced (Bamborough and Cummings 2009).

Interestingly, many hyperaccumulators used to follow definite strategy to amass specific bacteria resistant to particular metal(s) around their roots. Plants like *Alyssum bertolonii*, *A. serpyllifolium* subsp. *lusitanicum*, *Sebertia acuminata*, or *Thlaspi caerulescens* subsp. *calaminaria* have been shown to host higher proportions of Cd-, Ni-, or Zn-resistant bacteria in the rhizosphere compared to non-hyperaccumulating plants or non-vegetated soil (Schlegel et al. 1994; Delorme et al. 2001; Mengoni et al. 2001; Lodewyckx et al. 2002; Becerra-castro et al. 2009). These plants gradually develop resistance to a set of metals. Likewise, higher proportions of different Ni-tolerant bacteria were found in the rhizosphere of *Alyssum serpyllifolium* subsp. *lusitanicum* when the plants are exposed to high Ni concentrations (Becerra-castro et al. 2009). A synergistic effect between plant roots and their associated bacteria is thus evident. Production of metabolites by bacteria is augmented by the indirect supply of necessary substrates in the root exudates provided by plants. On the other hand, bacteria at the root zone (plant growth promoting rhizobacteria, PGPR) may help in the production of phytohormones (such as indoleacetic acid (IAA), cytokinins, and ethylene) (Kidd et al. 2009). Further, development, physiology, and exudation of root are also stimulated by the weathering agents that improves nutrient uptake by plants

(Patten and Glick 1996; Gahoonia et al. 1997; Barker and Banfield 1998; Gamalero et al. 2002; Calvaruso et al. 2006; Kidd et al. 2009).

7.10 Selection of Plants and Enhancing the Efficacy of the Process

Improvement of biomass production is most important for the application of phytoextraction technology that results in a higher metal extraction or total metal yield. As for example, inoculation of rhizobacteria *Pseudomonas fluorescens* biotype F, isolated from heavy metal contaminated soil, helped to improve the growth of sunflower plants (*Helianthus annuus*) and their tolerance to arsenate in soil (Raab et al., 2005). Bacterial production of IAA and siderophores played important roles to develop tolerance towards arsenate (Prasad 2007). Few studies suggest that application of transgenic plants along with rhizospheric PGPR improve plant biomass that will help in phytoextraction (Farwell et al. 2006). *Pseudomonas putida* HS-2 (isolated from Ni-contaminated soil) applied to the transgenic canola (*Brassica napus*) showed trends of higher accumulation of total Ni per plant. However, Kuffner et al. (2008) reported that rhizobacterial strains which were found to increase Cd/Zn uptake and accumulation and consequently growth of *Salix caprea* were neither phytohormone-producing strains nor siderophore producers.

Application of bioremediation practices depend upon the detoxification of toxic metals and xenobiotics through metabolism. It is reported that among various molecules, proteins like cytochrome P450, phytochelatins, and metallothioneins are very important biomolecules in this process. Augmenting the expression of these biomolecules may help to improve the efficiency of bioremediating agent (Choi et al. 1996; Clemens et al. 1999; Morris et al. 1999; Cobbett 2000; Cobbett and Goldsbrough 2002; Morant et al. 2003; Gillam 2008). Genetic supplementation by creating transgenic plants to increase remediation potential of highly toxic element is an alternative approach in this technology. It has been shown that tobacco plants carrying MerA gene from E. coli (encoding mercuric reductase) can mobilize mercury 5–8 times higher than control counterpart (Ke et al. 2001; Glick 2004). Similarly, over expressing two bacterial genes (encoding arsenate reductase (arsC) and γ-glutamylcysteine synthetase (γ-ECS)) in the small weed *Arabidopsis thaliana* significantly increased the accumulation of arsenic in leaves (Doucleff and Terry 2002). Reduction of arsenate to arsenite is catalyzed by the arsC, while γ-ECS catalyzes the first step in the synthesis pathway of phytochelatins, increasing the pool of thiol compounds including phytochelatins, all through the body of the plant. After detoxification of arsenite by thiol compounds forming arsenic–protein thiolates, may be stored and/or partitioned in the vacuole enabling arsenic to accumulate at greater amounts in the leaves of these transgenic plants (Doucleff and Terry 2002; Dhankher et al. 2002; Michel et al. 2007).

Phytoremediation process, thus, may be improved using plant-associated microorganisms that alter the solubility, availability, and transport of trace elements and nutrients by reducing soil pH, secretion of chelators and siderophores, or redox changes. Selenium (Se) phytoremediation (accumulation and volatilization) by Indian mustard (*Brassica juncea*) was most effective in the presence of plant growth promoting rhizobacteria (de Souza et al. 1999). Available data suggests that bacteria such as *Azotobacter chroococcum* (N_2-fixer), *Bacillus megaterium* (P-solubilizer), and *Bacillus mucilaginosus* (K-solubilizer) and *Bacillus* sp. RJ16 can decrease soil pH, probably by excreting low weight molecular acids, enhancing the bioavailability of heavy metals like Cd and Zn for plants (Morant et al. 2003; Wu et al. 2006; Sheng and Xia 2006). It has been seen that the presence of different rhizobacteria associated with three plants, *Alyssum murale*, *A. serpyllifolium* subsp. *lusitanicum*, *Thlaspi caerulescens*, increased the potentiality of heavy metal accumulation to their bodies (Whiting et al. 2001; Cloutier-Hurteau et al. 2008; Becerra-Castro et al. 2009). Rhizosphere actinobacteria *Alnus glutinosa* living in symbiosis with N_2-fixing *Frankia* were found to tolerate more than 2.0 mM Ni along with the increase yield of the plant (Wheeler et al. 2001). Likewise, a bacterial mixture of bacteria *Microbacterium saperdae*, *Pseudomonas monteilii*, *and Enterobacter cancerogenus* helped in higher zinc extraction by plants like *T. caerulescens* (Delorme et al. 2001).

For wastewater treatment in wetlands, establishing a dense stand of vegetation is more important than selecting a particular species. Any species that will grow well can be chosen. However, for storm water wetlands, native plant species work best. Selecting native, local plant species for wetland restoration is required as the plants are adapted to the local climate, soils, and surrounding plant and animal communities, and are likely to do well (Fig. 7.2). As for example, Bulrushes (*Scirpus* sp.) are widely used in treating sewage and wastewaters due to their ability to withstand high levels of nutrients, establish easily and noninvasive nature. Like that, arrowhead (*Sagittaria* sp.) and pickerelweed (*Pontederia cordata*) may be used in agricultural wetlands. The efficiency of water hyacinth (*Eichhornia crassipes*) for nutrient uptake and their rapid growth rate have put them to use for many years in cleaning up municipal and industrial wastewater (Vesk et al. 1999; Lombi et al. 2000; Prasad et al. 2001; Prasad 2007; Espinoza-Quinones et al. 2009). Water hyacinth has been shown to accumulate trace elements and as the recycling process is run by photosynthetic activity and biomass growth, the process is sustainable and is also energy and cost efficient (Garbisu et al. 2002; Lu et al. 2004; Bertrand and Poirier 2005). Few aquatic plants, as mentioned in the Table 7.1, have already been identified for their potential role in the remediation of metal-contaminated areas (Prasad et al. 2001; Prasad 2007).

Fig. 7.2 Schematic representation of amelioration of industrial and urban wastewater in wetlands

Table 7.1 Plants for heavy metal phytoremediation (Prasad et al. 2001; Prasad 2007)

Azolla filiculioides	Cr, Ni, Zn, Fe, Cu, Pb
Ceratophyllum demersum	Cd, Cu, Cr, Pb, Hg, Fe, Mn. Zn, Ni, Co, and radionuclides
Elodea densa	Hg, methyl-Hg
Eichhornia crassipes	As, Cd, Co, Cr Cu, Al, Ni, Pb, Zn, Hg, P, Pt, Pd, Os, Ru, Ir, Rh
Lemna minor	Mn, Pb, Ba, B, Cd, Cu, Cr, Ni, Se, Zn, Fe
Ludwigia natans	Hg, methyl-Hg
Lysimachia nummularia	Hg, methyl-Hg
Nuphar lutea	Cu, Ni, Cr, Co, Zn, Mn, Pb, Cd, Hg, Fe
Nymphaea alba	Ni, Cr, Co, Zn, Mn, Pb, Cd, Cu, Hg, Fe
Nymphoides germinate	Cd, Cu, Pb, Zn
Potamogeton communis	Ni, Cr, Co, Zn, Mn, Pb, Cd, Cu, Hg, Fe
Potamogeton pectinatus	Mn. Pb, Cd, Cu, Cr, Zn, Ni, As, Se
Phragmites karka	Cr
Pistia stratiotes	Cu, Al, Cr, P, Hg
Pteris vittata	As
Ruppia maritima	Mn, Pb, Cd, Pb, Fe, Se
Scapania uliginosa	B, Ba, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Pb, Sr, V, Zn
Schoenoplectus lacustris	Ni, Cr, Co, Zn, Mn, Pb, Cd, Cu, Hg, Fe
Typha latifolia	Ni, Cr, Co, Zn, Mn, Pb, Cd, Cu, Hg, Fe
Wolffia globosa	Cd, Cr

7.11 Phytoremediation at East Calcutta Wetland: A Case Study

The East Calcutta Wetlands (ECW; latitude 22°33′–22°40′N; longitude 88°25′–88°35′E), a Ramsar site (no. 1208) and wetland international site (reference no. 2IN013), is a globally recognized conserved wetland area. The site receives untreated municipal and industrial wastewater of the city of Kolkata (previously known as Calcutta) for nearly the last 100 years through a web of wastewater carrying canals passing through this area (Chatterjee et al. 2010). The ECW is the biggest urban wetland ecosystem in India covering 125 square kilometers of area with salt marshes, sewage farms, and settling ponds. Sewage from the city of Kolkata is treated by this wetland, and the nutrients contained in the wastewater also sustain fish farms and agriculture. In ECW areas, solid municipal garbage and sludge-filled soils are used for agriculture. Between 2,000 and 3,000 metric tonnes of solid wastes, in different forms, are being deposited daily in the wetland areas. Garbage farming yields nearly 370,650 kg ha^{-1} per year, which amounts to 150 metric tonnes of vegetables daily. Huge composite industrial effluent mixed with city sewage (around 50,000 m^3 per day) from Kolkata city is discharged, without any pretreatment, to a number of canals. Most threatening was Cr contamination of the area by untreated effluents from different industries including 538 tanneries located at the eastern fringe of Kolkata city. Therefore, biota in the ECW ecosystem is thought to be under potential threat of hazardous metal contamination to human. The wetland plants of this region have taken a key role in ameliorating heavy metal contaminated soil and water. Metals namely, Cr, Cu, Pb, Zn, Mn, and Fe were studied for phytoextraction properties in ten different plants of the region (Chatterjee et al. 2011). It was found that plants like Bermuda grass (*Cynodon dactylon*) had the highest total Cr concentration (6,601 $\pm$ 33 mg kg^{-1} dw). In this study (Chatterjee et al. 2011), it was also found that the extent of accumulation of various elements in the plants of the study sites was Pb (4.4–57 mg kg^{-1} dw), Cu (6.2–39 mg kg^{-1} dw), Zn (59–364 mg kg^{-1} dw), Mn (87–76 mg kg^{-1} dw), Fe (188–8,625 mg kg^{-1} dw), Ca (969–3,756 mg kg^{-1} dw), and Cr (27–660 mg kg^{-1} dw) indicating an uptake gradient of elements by plants as Ca > Fe > Mn > Cr > Zn > Cu > Pb (Chatterjee et al. 2011). Again, metal accumulation and localization in the root are of interest for the physiology and ecology of plant survival under elevated metal levels. X-ray microanalysis study revealed the patterns of distribution of elements along the length of roots of plants, water hyacinth (*Eichhornia crassipes* (Mart.) Solms), and common arum (*Colocasia antiquorum*) confirming their ability to amass heavy metals in higher concentration (Chatterjee et al. 2007). Further, plant species like sunflower (*Helianthus annuus*), marigold (*Tagetes patula*), and cock's comb (*Celocia cristata*) grew on soil contaminated by industrial sludge and irrigated regularly with wastewater in the ECW were also examined for their potential role in the phytoremediation process. It was found that general accumulation patterns of metals concerned in different plant parts were root > leaf > stem > flower. Cultivation of economically important, nonedible, ornamental plant species is an alternative cost-effective practice to remediate heavily

contaminated areas. Further, among these three plants, the biomass produced by cock's comb (14.7 kg dw m^{-2} per year) was the highest followed by sunflower (8.3 kg dw m^{-2} per year) and marigold (4.1 kg dw m^{-2} per year). Hence, for the purpose of phytoremediation, the option might be to use high biomass producing plants that were also useful for economy of the area (Prasad 2006; Chatterjee et al. 2012).

7.12 Concluding Remarks

In a wetland, vegetative mass provided by the growing plants redirect flow of water and its rhizosphere region stabilizes substrates and provides attachment sites for microbial development. Rhizosphere in association with decaying plant biomass generates litter and liberates organic carbon to stimulate microbial metabolism. Potential conversion of the waterweeds harvested from the area may be used for the production of fuel, paper, fiber, and energy (Curtis and Duke 1982). Utilizing the plants at the wetlands for heavy metal remediation, persistent emergent plants like common reed (*Phragmites* sp.), bulrushes (*Scirpus* sp.), spikerush (*Efeocharis* sp.), sedges (*Cyperus* sp.), rushes (*Juncus* sp.), and cattails (*Typha* sp.) are suitable. These species are suitable for wastewater treatment as they are habituated to tolerate continuous flooding and exposure to wastewater containing relatively high and often variable concentrations of pollutants. Further, any local species can also be taken into consideration as those are adapted to the local climate, soils, contaminants, and surrounding plant and animal communities. Treating diverse contaminants including metals by a wetland, diverse assemblages of wetland plants is probably the best suitable option that usually recovers faster from sudden anthropogenic disturbances like rapid inputs of varied contaminants. These native plant assemblages are aesthetically pleasing and may perform well in resisting invasive species and pests. However, the evolutionary significance of the trends on metal-specific accumulation among plant species occupying the same general habitat is an interesting area for future research.

Handling and disposal of the contaminated plant waste is the major concern with the application of phytotechnology. Periodic harvesting of metal accumulated biomass and disposing as hazardous waste, involve added cost. However, a number of options are available like landfills, production of fuel, fiber, and energy for proper disposal of metal-rich plants. Thus phytoremediation, in combination with burning the biomass to produce electricity and heat, could become a new environmentally friendly form of pollution remediation (Peuke and Rennenberg 2005). Further, metals can be recovered from the ash (bio-ore) produced by incineration. It was reported that Zn and Cd recovered from a typical contaminated site could have a resale value of more than one thousand US dollar per hectare (Watanabe 1997). Nicks and Chambers (1998) reported that using the nickel (Ni) hyperaccumulator *Streptanthus polygaloides*, a yield of 100 kg ha^{-1} of sulfur-free Ni could be produced. Thus, phytomining is a potential technology, however, has only limited

potential application. The economic viability of phytomining will improve as the price of metals increases. The financial attractiveness of phytomining should increase, particularly if it can be combined with other technologies such as phytoremediation and biofuel production (Brooks et al. 1998; Sheorana et al. 2009).

Most appropriate strategy to take care of a specific site may be selected by considering three crucial principles: the possibility of the pollutant to convert into a less toxic form through biological transformation (biochemistry), the availability of the pollutants to microbial population (bioavailability), and the prospect for biological activity (bioactivity). The potential for the use of plants for the detoxification or phytoremediation of polluted wetland areas is being increasingly examined. Cutting-edge approaches like incorporation of specific CYP genes for detoxification of xenobiotics along with upregulation of chelating proteins like phytochelatins, metallothionein, and thus next generation of GM plants along with microbes might play an important role in the wide application of the green technology.

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Chapter 8
A Multi-disciplinary Challenge for Phytoremediation of Metal-Polluted Pyrite Waste

Teofilo Vamerali, Marianna Bandiera, and Giuliano Mosca

8.1 The Phytoremediation Concept Evolves in Pyrite

After the discovery of *hyperaccumulators* (Raskin et al. 1994; Salt et al. 1995), plants which accumulate high above-ground levels of one or a few metals without evident symptoms of toxicity, the application of plant-based technologies to remedy metal-contaminated soils has received huge attention. Metalliferous soils provide several hyperaccumulators, but their application must be verified carefully in terms of biomass—generally very small—and uptake when plants are cultivated out of their native environment (Brooks et al. 1977).

Phytoextraction consists of removing toxic elements through the harvestable biomass, after sufficient translocation from roots has occurred. Although promising, the method has some limitations due to difficult plant establishment, possible limited soil metal availability, insufficient root uptake (exclusion), symplastic mobility and xylem loading, as well as the great energy costs required for detoxification and storage (Meagher 2000; Clemens et al. 2002).

The use of biomass species (trees and crops) may represent a realistic alternative to hyperaccumulators for removing trace metals (Vamerali et al. 2010). Biomass species can absorb a wider range of metals but at low concentrations, a feature compensated by higher biomass productivity. The application of cultivated species is facilitated by the easy availability of seeds and cuttings on the market, but their adaptability and method of cultivation should be verified in each specific site. The extended root system of trees is suitable for remediating especially deep polluted layers (Pulford and Dickinson 2005) and short-rotation coppices of poplar (*Populus*

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spp.) (Laureysens et al. 2004a, b) and willow (*Salix* spp.) (Rosselli et al. 2003; Dickinson and Pulford 2005) may provide an efficient and cost-effective decontamination method. Herbaceous species produce a denser vegetation cover which is effective against erosion and may create an aerobic environment in the rhizosphere, increasing soil aggregation and binding contaminants through the release of organic matter (Pulford and Watson 2003; Robinson et al. 2006). Roots can also efficiently act in phytostabilisation by sequestering metals, especially those with limited mobility such as Pb and Cu (Marmiroli et al. 2005; Yoon et al. 2006) and favouring precipitation with root exudates (Heim et al. 1999; Yang et al. 2005). Various means have been successfully tested in the last 10 years to improve phytoextraction efficiency in biomass species, but mainly in agricultural or forest soils. Assisted phytoextraction with low toxic organic chelators, like NTA (nitrilotriacetic acid) and EDDS (ethylene-diamine-disuccinic acid), positively increase metal uptake in Ethiopian mustard (*Brassica carinata* A. Braun) (Quartacci et al. 2007). Exogenous applications of growth regulators may also result in higher growth and metal uptake in alfalfa (*Medicago sativa* L.) and sunflower (*Helianthus annuus* L.) (Lopez et al. 2005; Liphadzi et al. 2006), whereas mycorrhization facilitates metal acquisition in maize (*Zae mays* L.) (Shen et al. 2006; Wang et al. 2007). However, investigations are often conducted in the laboratory or in microcosms, thus making transferral of results to the open field ineffective. Only few experiments have been carried out *in situ* and limited information is available on particular substrates, such as sediments and industrial wastes. In this framework, a summary of results on the phytoremediation of pluri-metal-contaminated pyrite cinders is presented here, focusing on plant responses to several agronomic practices at pot and field level. As a single green technology may fail in this context, the traditional concept of phytoremediation should be reviewed in the light of a multidisciplinary approach.

8.2 What the Literature States on Phytoremediation of Pyrite

Among metal-polluted media, great concern focuses on industrial waste or sediments, the unusual composition of which may further limit plant establishment and growth. Among these, we considered pyrite waste, which remains after sulphur extraction from pyrite ore roasting at extremely high temperatures (~800 °C). The waste presents itself as red cinders, mainly composed of fine particles of pyrite (FeS_2) and other minerals and devoid of organic matter (Vidal et al. 1999). Oxidation of metal sulphides from pyrite residues can release soluble metals and increase soil acidity (Clemente et al. 2006), with consequent hazardous metal movements. Phytomanagement of pyrite waste is an interesting and inexpensive option to reduce wind erosion and metal leaching, but little information is available in the literature on this issue, particularly at field scale. In recent years, some authors have found that cultivation of soybean (*Glycine max* (L.) Merr.), sorghum (*Sorghum bicolor* L.), maize and sunflower is possible at various rates of pyrite dilution, but only at pot level (Fellet et al. 2007). In the open field, the establishment and

phytoremediation of Indian mustard (*Brassica juncea* (L.) Czern) in a site affected by the toxic spill of pyrite residues at Aznalcóllar (Spain) was effective only after the addition of organic matter (cow manure and compost) and amendment with lime to increase the pH (Clemente et al. 2006). Substantial biomass increases have also been achieved with sunflower and sorghum after organic or mineral fertilisation of pyrite waste (Marchiol et al. 2007), suggesting that attention should be paid to improvements in the physical and chemical properties of the substrate.

In both pyrite and other metal-contaminated wastes, plant growth is limited not only by contamination but also by several environmental variables, such as unsuitable pH, high salinity, insufficient aeration and low water and nutrient availability (Robinson et al. 2006). In these conditions, extensive root colonisation is essential for plant establishment and metal acquisition, but root responses under metal contamination have been investigated in a narrow range of species. For instance, roots of the hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. were found to colonise predominantly Zn-polluted soil regions (Saison et al. 2004), whereas little information is available for most biomass species. The root system of non-metallophyte species is expected to be very sensitive to the presence of metals, with serious damage and growth reduction (Ubi and Osodeke 2007; Rascio et al. 2008). For instance, disruption of the root cuticle, reduced root hair proliferation and severe deformation of root structures are caused by copper in *Chloris gayana* Kunth (Sheldon and Menzies 2005). According to a general rule, which recommends thorough analysis of polluted sites before the application of any phytoremediation strategy (Wiegleb and Felinks 2001), the area contaminated by pyrite waste and metals which we studied was initially characterised for soil stratigraphy, contaminant distribution and floral analysis.

8.3 Site Characterisation

We focused attention on a contaminated area at Torviscosa (Udine—NE Italy, 45°49′23″N, 13°16′40″E, 3 m a.s.l.), near an abandoned chemical factory and within the polluted site 'Lagoon of Grado and Marano and adjacent rivers', which is included in the Italian priority site list for remediation (Fig. 8.1). Pollution was due to As- and metal-contaminated pyrite cinders, discharged between the 1940s and the late 1970s as by-products of pyrite ore roasting for sulphur extraction. Largely devoid of organic matter, with high bulk density (1.65 g cm^{-3}), poor in nutrients, pH 7.3 (Table 8.1) and relatively low electrical conductivity (0.3 S m^{-1}), over the years the cinders had been colonised by sparse spontaneous flora (Coletto et al. 2006). Within a confined area of 2,000 m^2 of the site, total metal concentrations in the substrate were identified in 2004 in 33 soil samples (2 m deep, 10 m apart); soil stratigraphy was monitored by the digging of six exploratory ditches (Fig. 8.2). Metals were detected in substrate samples and plant tissues by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy) after microwave-acid digestion. The cinders extended for a depth of 0.7 m over a deep

Fig. 8.1 Polluted site at Torviscosa (Udine, NE Italy). (**a**) Yellow deposit of sulphur on soil surface near abandoned factory. (**b**) Exploratory ditch (1.5 m) for studying soil/pyrite stratigraphy. (**c**) Detail of gravelly capping soil with aged spontaneous vegetation (in winter). (**d**) Dense vegetation of *Pyracantha coccinea* M.J. Roemer (in winter) close to old factory

clay horizon and were capped with ~0.15 m of unpolluted gravelly soil. In detail, analysis of soil stratigraphy showed five different layers with the following characteristics:

a. 0–0.15/0.20 m: carry-over soil with vegetation, rich in gravel, with sand and silt
b. 0.15/0.20–1.20/1.75 m: wet pyrite cinders
c. 1.20/1.75–1.40/2.35 m: wet black–brown silty clay, rich in organic matter
d. 1.40/2.35–1.85/3.20 m: wet clay with grey–green silt
e. 1.85/3.20–2.90/3.80 m: white sandy soil with fine gravel, very wet (groundwater)

The presence of the almost impermeable clay horizon (layer *d*) has prevented significant downward metal leaching until recently, as confirmed by groundwater analysis. The high soil moisture along much of the profile, together with extended waterlogging during rainy periods, was indeed partly due to poor water infiltration in the cinders.

Table 8.1 Main chemical properties and level of metal contamination ($n = 3$) of pyrite waste and *in situ* capping soil, in comparison with a silty-loam uncontaminated reference soil (Legnaro—University of Padova)

	pH	O.M.[a] %	N[b] g kg^{-1}	P[c] mg kg^{-1}	K[d] mg kg^{-1}	As[e] mg kg^{-1}	Cd[e] mg kg^{-1}	Co[e] mg kg^{-1}	Cu[e] mg kg^{-1}	Pb[e] mg kg^{-1}	Zn[e] mg kg^{-1}
IGV[f]						20	2	20	120	100	150
Unpolluted capping layer	8.1	1.87	0.73	11.8	17.3	19.9	0.83	<0.01	72.8	22.4	96.5
Pyrite waste	7.3	<0.01	n.d.	n.d.	n.d.	**892**	**5.09**	**102**	**2,726**	**459**	**2,410**
Mixed soil-pyrite											
Ploughing[g]	7.6	2.52	5.33	12.7	153	**243**	**2.67**	**41.6**	**1,719**	**118**	**860**
Ploughing[h]						n.d.	0.31	n.d.	291	15.7	30.2
Ripping[g]	7.5	2.85	6.67	7.90	131	**292**	1.67	**29.5**	**1,103**	89.8	**674**
Ripping[h]						n.d.	0.16	n.d.	190	10.3	24.1
Reference soil at Legnaro[g]	7.8	2.17	5.67	33.1	62.8	15.5	0.38	7.95	31.1	17.9	79.5
Reference soil at Legnaro[h]						n.d.	0.09	n.d.	4.51	2.96	1.21

Values in bold exceed IGV[f]

n.d. not determined

[a]Organic matter: Walkey and Black method

[b]Total N: Kjeldhal method

[c]Available P: Olsen method

[d]Exchangeable K: $BaCl_2$ TEA (ISO 11260)

[e]USEPA 3051 method

[f]Italian Guideline Values for 'Green public, private and residential areas' (Italian Legislative Decree 152/2006)

[g]Total metal concentration after tillage (0–0.15 m of depth)

[h]DTPA-extractable metal concentration after tillage (0–0.15 m of depth)

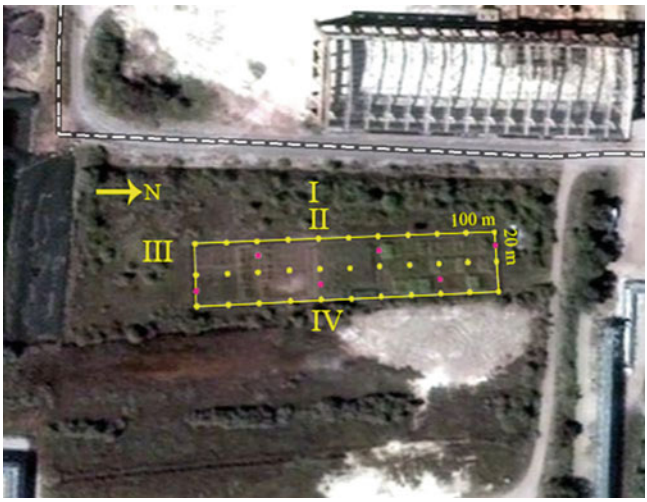

Fig. 8.2 Aerial view of study area. *Yellow dots*: soil sampling (33); *red dots*: exploratory ditches (6); *Roman numbers* (I–IV): areas for floral analysis. *Arrow*: north

The carry-over topsoil (layer *a*) generally had low metal contents, below the Italian Guideline Values (IGV) for 'Green public, private and residential areas' (Italian Legislative Decree 152/2006). Metal concentrations in cinders were heterogeneous across the sampling area, but on average very high, exceeding the IGV by as much as 45 times for As, 23× for Cu, 16× for Zn, 5× for Co and Pb, and 2.5× for Cd. Arsenic and Cu levels were particularly high and exceeded the less restrictive IGV for 'Industrial sites' (i.e., 50 and 600 mg kg^{-1} DW, respectively) (Table 8.1). The total amounts of Fe and S in the cinders were about 10 and 5 times higher than in cultivated soil, with concentrations of 97 % and 0.39 %, respectively. In spite of this, bioavailable Fe was not very high, comparable with the agricultural silty-loam soil at the experimental farm of the University of Padova (24 vs. 18 mg kg^{-1}).

The particular stratigraphy, together with abundant precipitation—the historical mean annual value of the site is 1,000 mm—led to the selection of a specific spontaneous flora. Analysis of the vegetation cover by visual evaluation (Pignatti and Mengarda 1962) during spring 2004 in four buffer zones identified in the surroundings of the soil-sampled area (Fig. 8.2) was believed to be useful in providing criteria for species selection for the planned phytoremediation setting. Buffer zones I and IV were colonised by both herbaceous and woody species, whereas the vegetation was mainly herbaceous in buffer zones II and III. For species with an appreciable degree of cover (>5 %), shoot samples (young branches for trees) were collected in early spring, washed and oven-dried (105 °C, 24 h) to determine metal concentrations. Our hypothesis was that a correlation exists between the extent of species diffusion and their metal accumulation.

In the buffer zones, more than 80 different species were classified, mainly herbaceous and only 10 woody. In zone I, close to the old factory, the latter were mainly represented by *Pyracantha coccinea* M.J. Roemer (27.5 %, i.e., percentage of the sum of all detected species), *Salix* spp. (21.6 %) and *Populus alba* L. (8 %). The most widespread grasses were *Solidago gigantea* Aiton (8 %) and *Dorycnium pentaphyllum* Scop. (8 %). Dominant species in zones II and III were *Poa pratensis* L. (24 % and 11.4 %, respectively), *Ambrosia artemisiifolia* L. (18 % and 34 %), *Medicago lupolina* L. (6 % and 9 %) and *Bromus arvensis* L. (11 %, only in zone III). Lastly, in zone IV the prevailing species were *Phragmites australis* (Cav.) Trin. ex Steud. (15 %), *Solidago gigantea* Aiton (10 %), *Dactylis glomerata* L. (7 %) and *Populus alba* L. (10 %).

Zinc, Mn and Cu were the three most frequently accumulated elements in the shoot tissues of all species, both herbaceous and woody (Fig. 8.3). The highest values of Zn and Cu were found in *Taraxacum officinale* Weber (360 and 96 mg kg^{-1}, respectively) and Mn in *Carex hirta* L. (393 mg kg^{-1}). The overall metal concentrations (summation of various elements) were highest in *Asteraceae* species, i.e., *T. officinale*, *Eupatorium cannabium* L. and *A. artemisiifolia* L., the latter being the most widespread. Interesting accumulations were also found in the hydrophyte *C. hirta* (family *Cyperaceae*), whereas trees and shrubs seemed to be less efficient than herbs, except for the *Salicaceae Salix alba* L. and *Populus nigra* L., which have been found to accumulate Zn efficiently in this and in other contaminated sites (Rosselli et al. 2003; Pietrini et al. 2010). These preliminary investigations confirmed the importance of species selection in phytoremediation. Although a particular relationship between metal accumulation and kind of root apparatus does not seem to exist (Fig. 8.3), the ability of our *Asteraceae* may partially depend on their deeper tap roots. The application of spontaneous species still raises the problem of seed supply and, with this in mind, screening of cultivated species was considered necessary.

8.4 Experience in an *On-Site* Pilot Phytoremediation Plant

The sparse natural vegetation cover of the site meant that difficulties in plant establishment and growth were predicted, but the presence of the capping unpolluted layer seemed useful for the vegetation. In a preliminary pot trial, we verified whether some crops like sunflower, alfalfa and fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.) could take advantage of a 7- or 15-cm top unpolluted soil layer (Fig. 8.4). Indeed, mimicking site stratigraphy, regardless of the thickness of the capping layer, all species showed regular growth both above- and below-ground over a 60-day period of cultivation, comparable with that of the uncontaminated reference soil of the University of Padova. However, roots tended to colonise mainly the uncontaminated layer (length: 90 % vs. 80 % of pyrite alone and 50 % of controls). The general marked reduction in plant growth with pyrite alone was evident, i.e., −77 % in shoots and −63 % in roots (length) on average.

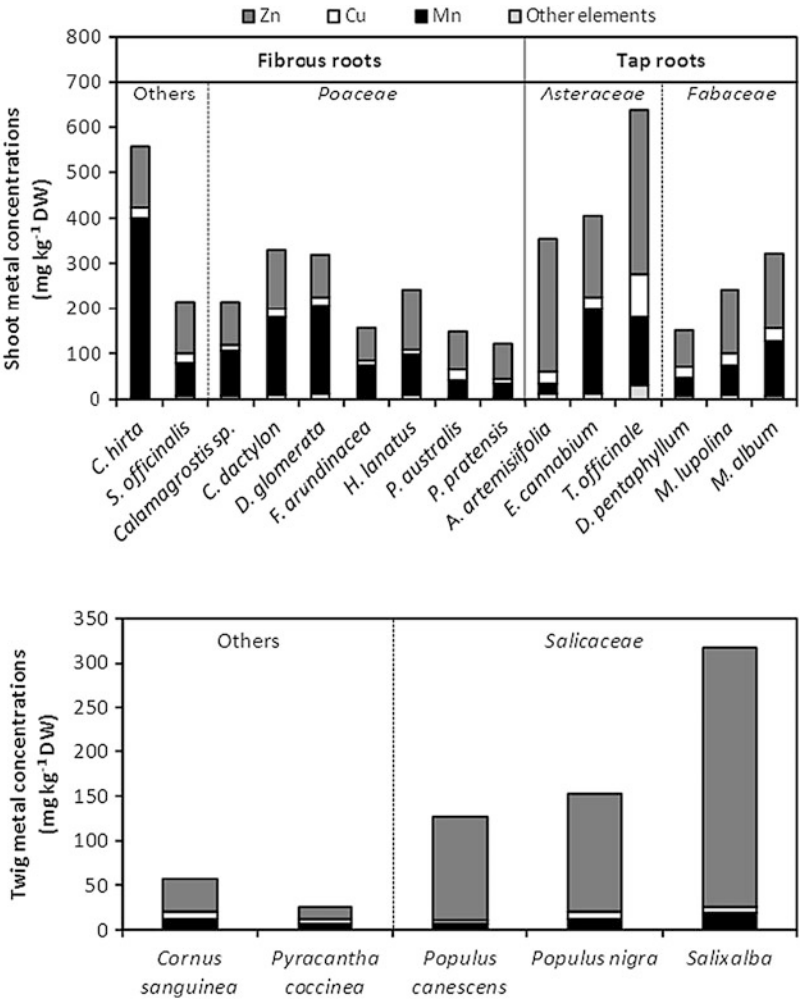

Fig. 8.3 Metal concentrations (mg kg^{-1} DW) in shoots of various spontaneous herbs (*above*) and wood of trees (*below*) collected at pyrite-contaminated site, sorted by root system type and botanical family. Herbs studied: *Carex hirta* L., *Sanguisorba officinalis* L., *Calamagrostis* sp., *Cynodon dactylon* (L.) Pers., *Dactylis glomerata* L., *Festuca arundinacea* Schreb., *Holcus lanatus* L., *Phragmites australis* (Cav.) Trin., *Poa pratensis* L., *Ambrosia artemisiifolia* L., *Eupatorium cannabium* L., *Taraxacum officinale* Weber., *Medicago lupolina* L., *Melilotus album* Desr. Other elements: As + Cd + Co + Cr + Ni + Pb

Setting up the phytoremediation plant at Torviscosa in 2005 gave us the opportunity of testing various soil management strategies, by comparing unaltered stratigraphy with mixed layers, i.e., ripping vs. ploughing tillages, both at a depth of 0.3 m. Ploughing entailed more thorough mixing of soil than ripping, which simply broke up the surface. Ploughing was intended to dilute the waste with the unpolluted top soil, and ripping to allow roots to encounter a clean habitat, at least

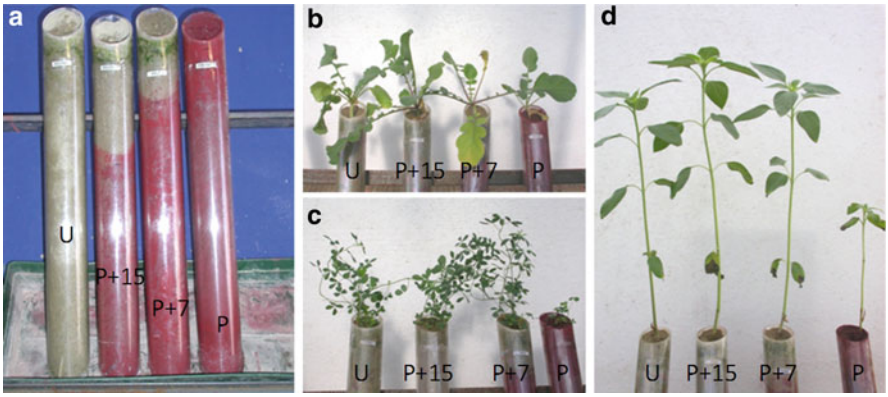

Fig. 8.4 Aspect of pyrite (**a**) and crop species (**b**, **c**, **d**) after 60 days of cultivation in rhizoboxes under various treatments. U: uncontaminated reference silty-loam soil; P+15: pyrite cinders capped with 15 cm of unpolluted soil; P+7: pyrite cinders capped with 7 cm of unpolluted soil; P: pyrite cinders

initially. In this way, in the sowing bed (top 0.15-m layer) pollution was roughly halved by ploughing and, despite some upward cinder movement, ripping led to lower contamination than ploughing (total and DTPA-extractable: ~−30 %) (Table 8.1).

Given the low fertility of pyrite (Marchiol et al. 2007, personal comunication in 2005), before sowing 100 kg ha^{-1} of each N, P_2O_5 and K_2O as chemical fertilisers were incorporated into the soil by harrowing. Four crop species, i.e., sunflower, Italian ryegrass (*Lolium multiflorum* Lam.), alfalfa and fodder radish (Vamerali et al. 2011b), and four woody species, i.e., white poplar (*Populus alba* L.), black poplar (*P. nigra* L.), European aspen (*P. tremula* L.) and white willow (*Salix alba* L.) (Vamerali et al. 2009), were grown under the two soil tillages and compared with the ploughed uncontaminated soil reference of the experimental farm of the University at Legnaro (45°21′N, 11°58′E, 12 m a.s.l.). Sowing of crops and transplanting of 2-year-old bare rooted cuttings of woody species took place in May, and shoot (biomass) and root investigations (RLD, volumetric root length density, by auger sampling) at the end of July and in mid-September, in both groups of species respectively.

Pyrite waste was an inhospitable substrate for all plants, as also reported by Fellet et al. (2007) and Marchiol et al. (2007), at the same site for other species. The anomalous physical properties (high bulk density and low water infiltration), together with high Fe and S, and multiple contamination of pyrite, greatly limited plant growth, almost regardless of tillage system. Only fodder radish profited by the lower contamination of ripping (Fig. 8.5). Improvements in the habitat should involve soil drainage and adequate irrigation, as we accomplished by digging shallow drains and setting up a low-intensity sprinkling system in summer.

The lower contamination due to ripping seemed to be less favourable for metal concentration in plants but more useful for growth, especially in fodder radish.

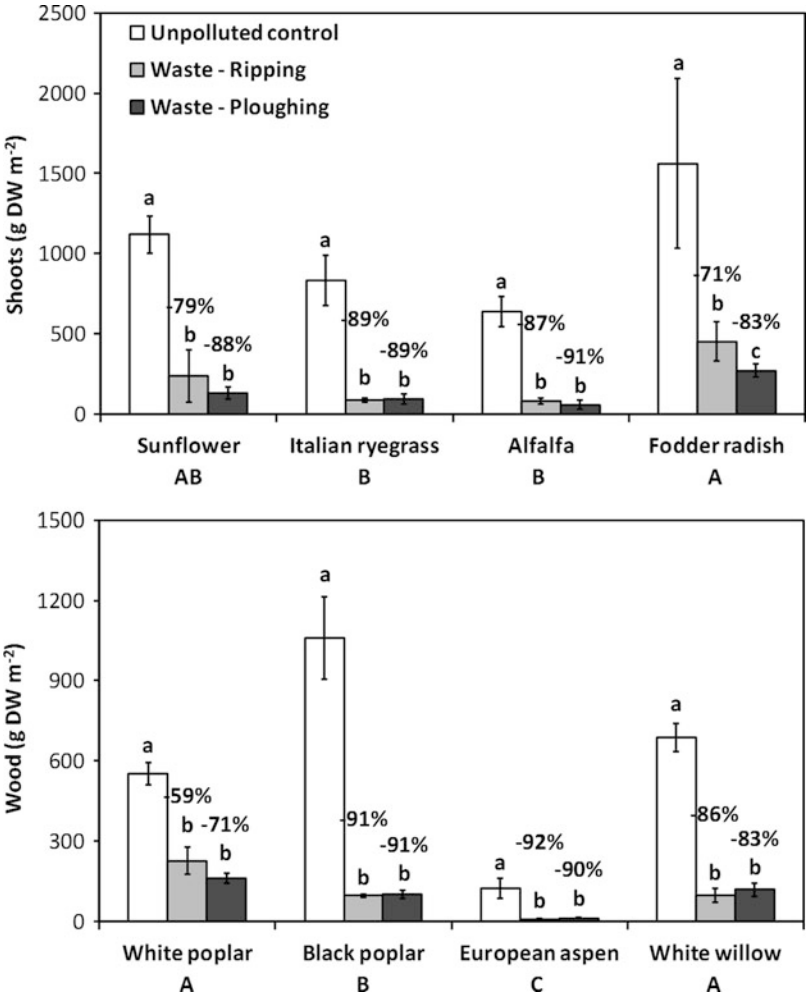

Fig. 8.5 Above-ground biomass (± S.E., $n = 3$) of crops at flowering (*above*) and trees (without leaves) 6 months after transplant (*below*) in pyrite waste under two tillages, in comparison with uncontaminated ploughed controls. Percentage of reduction for ploughing and ripping against control shown above bars. *Small letters*: statistically significant differences between treatments within same species (Newman-Keuls test, $P \leq 0.05$). *Capital letters*: statistically significant differences between species for pyrite only (main effect)

Overall, tillage choice was not critical in terms of mass balance of phytoextraction (Table 8.2), although we do recommend ripping to guarantee a better canopy cover against pollutant dispersion and for easier mechanical management of biomasses, e.g., cutting and harvesting operations. Among crops, fodder radish and sunflower were the highest biomass-yielding species, the former reaching the greatest but still poor metal removals (330 g ha^{-1} of metals). Fodder radish belongs to the

Table 8.2 Metal concentrations ($n = 3$) and removals in crop shoots and tree twigs of species under two tillage systems (Newman-Keuls test, $P \leq 0.05$)

Treatment	Species	As (mg kg^{-1})		Cd (mg kg^{-1})		Co (mg kg^{-1})		Cu (mg kg^{-1})		Pb (mg kg^{-1})		Zn (mg kg^{-1})		9 HM[(a)] (mmol kg^{-1})		Removals[(b)] (9 HM)	
Crops																	
Ploughing	Sunflower	0.15	c	0.42	a	0.19	c	14	b	0.48	c	89	a	1.9	a	175	a
	Italian ryegrass	3.9	b	0.42	a	0.41	ab	30	a	1.7	a	160	a	3.4	a	243	a
	Alfa-alfa	6.9	a	0.45	a	0.52	a	30	a	2.2	a	119	a	2.7	a	93	a
	Fodder radish	3.3	b	0.55	a	0.29	bc	25	ab	1.1	b	75	a	1.8	a	327	a
	MEAN	3.6	A	0.46	A	0.35	A	25	A	1.4	A	111	A	2.5	A	210	A
Ripping	Sunflower	n.d.		0.15	b	0.05	a	11	a	0.13	b	34	b	0.94	b	156	a
	Italian ryegrass	5.4	a	0.58	a	0.58	a	37	a	2.3	a	126	a	3.0	a	180	a
	Alfa-alfa	4.2	a	0.33	ab	0.37	a	23	a	1.42	ab	64	b	1.7	ab	90	a
	Fodder radish	3	a	0.27	ab	0.21	a	18	a	0.58	ab	48	b	1.2	b	334	a
	MEAN	3.2	A	0.33	A	0.30	A	22	A	1.1	A	68	B	1.7	B	190	A
Trees																	
Ploughing	White poplar	–		0.41	a	0.07	a	4.9	bc	–		62	b	1.2	b	12	a
	Black poplar	–		1.2	a	0.02	a	4.0	c	–		77	b	1.4	b	9.1	a
	European aspen	–		1.2	a	0.15	a	9.1	a	–		106	a	2.1	a	1.6	b
	White willow	–		0.52	a	0.09	a	7.6	ab	–		57	b	1.1	b	7.9	a
	MEAN			0.82	A	0.08	A	6.4	A			75	B	1.5	A	7.7	A
Ripping	White poplar	–		0.51	b	0.09	a	4.4	b	–		65	b	1.3	b	18	a
	Black poplar	–		1.6	a	0.09	a	4.3	b	–		103	a	2.0	a	12	ab
	European aspen	–		1.1	b	0.21	a	6.6	a	–		116	a	2.2	a	1.3	b
	White willow			0.77	b	0.14	a	7.5	a	–		94	ab	1.8	ab	11	ab
	MEAN			1.0	A	0.13	A	5.7	A			94	A	1.8	A	10	A

Small letters: differences among species within same tillage. Capital letters: differences between tillages (main effect) within each metal

[(a)]As + Cd + Co + Cr + Cu + Mn + Ni + Pb + Zn

[(b)]g ha^{-1} for crops and mg per plant for trees

Brassicaceae, a botanical family which should be exploited in phytoremediation as it also includes several hyperaccumulators (Krämer 2010). The biomass reached by radish in our waste was obviously poor when compared with the uncontaminated reference. Its productivity was similar to that of the small-biomass yielding hyperaccumulator *T. caerulescens* (Anderson et al. 1999), but it is expected to have greater efficiency in pluri-contaminated sites. Indeed, fodder radish has been used to good effect in our past experiments in a pluri-contaminated agricultural soil near Milan, showing better shoot and root growth than controls and better phytoextraction than other *Brassicaceae*, such as Indian mustard, oilseed rape (*B. napus* L. var. oleifera D.C.) and garden cress (*Lepidium sativum* L.) (Mosca et al. 2004). The behaviour of fodder radish below-ground was favourable; its RLD in the 0.3-m layer being similar to that of controls. There was no significant 'species × tillage' interaction and, unfortunately, much of the root length was confined to the top 0.1-m; root distribution was similar between tillages in sunflower and alfalfa, whereas fodder radish and Italian ryegrass positively moved a greater fraction of length (~15 %) downwards (below 0.1 m depth) with ripping.

At this point, the question which arose was: which are the criteria allowing selection of efficient herbaceous species in pyrite? A partial answer comes from the morphological features of roots. For instance, better translocation of metals from roots to shoots were related to high specific root length ($R^2 = 0.21$), whereas the maximum root length (Italian ryegrass), although correlated with shoot metal concentrations ($R^2 = 54$ %), is probably an invalid criterion when considered alone. The greater relative RLD and above-ground productivity with respect to the reference soil, as occurred in fodder radish, seem to provide better all-round criteria. The response of woody species was also variable in terms of growth and metal accumulation. Compared with controls, the wood production of white poplar was much less affected than that of black poplar (−65 % vs. −91 %), with small differences between tillages (Fig. 8.5). In general, As and Pb had not accumulated in twigs, Cu was low and Co was just above the detection limit (Table 8.2). Our tree species confirmed initial observations on spontaneous *Salicaceae*, only Zn having interesting levels, especially after ripping, i.e., 94 vs. 75 mg kg^{-1} of ploughing (means). We detected a negative correlation between biomass and concentrations of metals, so that white poplar yielded the best biomass and European aspen the highest metal concentrations. Also for trees, the expected removals were basically poor, although more precise phytoextraction balances depend on plant density of short-rotation coppices and rotation cycle.

When working on pyrite improvement, the first requirement is undoubtedly increased yields and, secondly, increased metal concentrations and translocation. Substantial productivity gains can be achieved with abundant fertilisation (Marchiol et al. 2007) and small plant sizes suggest that plant density could be increased, at least for wide-spaced crops like sunflower. Grasses could not tolerate frequent cutting in the contaminated area because of insufficient growth after the first harvest, as in Italian ryegrass and alfalfa. Undoubtedly, the latter was disadvantaged by the absence of root nodulation with *Rhizobium* and by particularly high shoot As, like *Salicaceae,* which lacked mycorrhization (data not shown).

Generally the roots of crops were less restricted in their growth than shoots, although roots had much higher metal concentrations, i.e., Cd 4-fold, Zn 7×, Co 28×, As 33×, Pb 51× and Cu 77×. They acted as a substantial accumulation sink for most trace elements, particularly Cu, Pb and As—a result also found in coarse and particularly fine roots of poplars and willow. Fine root biomass was quite modest, estimates from soil cores in crops not exceeding 20 g m^{-2}, and their contribution to long-term metal stabilisation is probably negligible because of fast turnover (Goins and Russelle 1996). For the coarse roots of trees and tap roots of annual species, degradation is probably slower, but this is an issue to be further investigated in phytostabilisation processes. From several aspects, root systems may hold the key to understanding the possibilities and options for phytomanagement of pyrite waste, although the maximum rooting depth (0.3 m) still remains to be greatly enhanced. In this context, the very high number of spontaneous species with shallow fasciculate roots is likely the result of severe selection of tap-rooted ones. Phytoextraction enhancement through increased metal concentrations in biomasses (e.g., by soil ploughing) turned out to reduce species differences, a strategy contrasting with many other agricultural practices which are instead addressed to yield improvements. The main information obtained from this trial was the need to reduce soil contamination through soil amendment and to facilitate plant establishment—for instance with the application of growth regulators.

8.5 Improving Pyrite Hospitality and Plant Metal Uptake

In order to improve the phytoextraction in pyrite, some pot trials with fodder radish were set up in 2006 and 2007, the aim being to improve the environment for roots and enhance above-ground productivity. In all experiments, plants were grown for 3 months in cylindrical 52-cm high pots (1.3 L volume), filled with a pyrite cinder–sand mixture and regularly watered with 50 % diluted Hoagland solution. Sand was added to attenuate contamination and improve water drainage, but leachates were collected in order to check whether our treatments had environmental counter-indications. Treatments were compared with untreated controls with five replicates.

We first thought of humic acid treatment for plants and pyrite directly. Humic acids (HA) are characterised by acidic groups which play an important role in enhancing the solubility, bioavailability, uptake and transport of metals (Evangelou et al. 2004), and are known for their auxin-like effect (Delfine et al. 2005). HA came from a commercial product (Humic super, Tiller—Italy) as liquid formulation (10 % DW of HA) and were applied as follows: foliar spraying (0.1 g HA L^{-1} solution, once a week for 3 weeks), two doses mixed with the substrate before sowing (0.1 and 1 g HA kg^{-1}) in combination or not with foliar treatment and a low rate (0.1 g HA kg^{-1}) applied at sowing through irrigation. The highest amendant dose of 1 g kg^{-1} positively increased shoot metal concentrations (overall elements: +44 %) but, unfortunately, curbed shoot growth and worsened metal removals

probably as consequence of high metal availability in pyrite (Bandiera et al. 2009). Improved translocation of all metals was the only positive effect of high HA dosages. Actually, only at the small dose of 0.1 g HA kg^{-1} were there significant increases in root length (+46 %) and—although with only slight above-ground yield improvements—substantial enhancement of plant metal removals (+35 %). These results confirm the auxin-like properties of humic substances, and their effectiveness at low rates is a premise for low-cost large-scale applications.

More complicated was management of exogenous application of IBA, one of the most powerful root-enhancing phytohormones. Starting from about 1 month after sowing, we tentatively applied IBA five times (at 10-day intervals) to fodder radish leaves at 10 mg L^{-1} or to the waste at 0.1 and 1 mg kg^{-1}, in association or not with foliar spraying. We obtained negative responses from this trial as—with the exception of foliar spraying alone—the hormone reduced shoot and root biomass (−60 % on average) when applied to the waste, probably due to unsuitable dosages and long persistence caused by low microbial activity (Vamerali et al. 2011a). The expected phytoextraction balance was thus greatly worsened, in spite of improvements in concentrations due to the chelating effect of this phytohormone.

Lastly, verification of the applicability of chelant-assisted phytoextraction was tested on pyrite, which is an uncommon substrate for this technique. We wished to ascertain whether the recently available EDDS (ethylene diamine disuccinic acid), characterised by higher degradability compared with EDTA (ethylene diamine tetracetic acid), could improve metal uptake without causing substantial phytotoxicity and leaching. The tested plants were fodder radish and Ethiopian mustard treated with [S,S]-EDDS at various doses and application times: 2.5 and 5 mmol kg^{-1} substrate applied through irrigation 1 week before harvest (common application time of chelators) and 1 mmol kg^{-1} soil repeated five times at 5- or 10-day intervals, respectively starting 48 or 28 days after sowing. At these doses, the chelator did successfully improve Cu, Co, Zn and Pb above-ground concentrations (Table 8.3), together with Cu translocation, but reduced plant biomass, especially with repeated applications and in radish (Bandiera et al. 2010). This may have a direct effect on leaching, as the drop in transpiration caused by diminished leaf area leads to significant losses of Cu, the metal with the greatest stability constant with EDDS (Tandy et al. 2004). Better metal phytoextraction (+31 %) together with minimal metal leaching was achieved with moderate (2.5 mmol kg^{-1}), traditional close-to-harvest chelator applications, but in Ethiopian mustard only. Certainly, these results on the use of EDDS and its management require on-site confirmation, but the generally unfavourable phytoextraction balance, associated with the uncertain fate of metal-EDDS compounds after plant harvest, gives rise to doubts about its use.

8.6 Conclusions

Phytoremediation of pyrite waste is complicated to manage because of multiple constraining factors which affect plant growth, beyond metal contamination. Removal of the most labile fraction of metals with field crops seems to be a feasible

Table 8.3 Shoot metal concentrations (mg kg^{-1}, $n = 5$) and translocation factor (shoot-to-root metal concentration ratio %) in two species under different EDDS treatments in pyrite

	As		Cd		Co		Cu		Pb		Zn	
	mg kg^{-1}	TF	mg kg^{-1}	TF	mg kg^{-1}	TF	mg kg^{-1}	TF	mg kg^{-1}	TF	mg kg^{-1}	TF
Ethiopian mustard												
C	4.6 a	8.38	0.28 b	74.7	0.14 c	3.02	29 c	11.6	-	-	80 c	16.2
2.5	5.4 a	7.70	0.43 ab	68.6	0.23 bc	3.03	49 b	19.2	-	-	99 ab	18.0
5	**5.9** a	6.67	0.48 ab	70.2	0.31 b	3.90	62 b	20.4	**0.84** a	0.23	**114** a	19.1
1 × 5–5d	5.6 a	5.90	**0.62** a	84.7	**0.62** a	8.93	**100** a	21.0	0.51 ab	0.09	103 ab	16.9
1 × 5–10d	5.1 a	7.26	0.58 a	131	0.33 b	6.34	55 b	15.7	0.25 ab	0.02	96 bc	15.3
Fodder radish												
C	13 a	14.8	0.76 a	85.9	0.28 b	2.67	39 b	9.58	2.2 b	1.12	91 a	17.4
2.5	16 a	28.8	1.30 a	167	0.47 b	7.53	90 b	17.6	3.8 b	1.96	102 a	8.75
5	12 a	23.0	0.80 a	150	0.73 ab	12.9	94 b	24.8	**8.1** a	1.11	**112** a	19.7
1 × 5–5d	**20** a	24.8	**1.60** a	202	**1.30** a	22.7	**161** a	28.5	2.5 b	0.30	107 a	15.9
1 × 5–10d	15 a	19.2	0.77 a	99.6	0.79 ab	12.7	87 b	19.2	1.3 b	0.05	95 a	10.2

C: untreated controls; 2.5 and 5: 2.5 and 5 mmol EDDS kg^{-1} substrate applied 1 week before harvest; 1 × 5–5d and 1 × 5–10d: 1 mmol kg^{-1} substrate repeated five times at 5- or 10-day intervals. Letters: differences among treatments within same species (Newman-Keuls test, $P \leq 0.05$). Highlighted values (bold) are the highest concentrations for a particular treatment in each species

phytomanagement option with some species and for some trace elements only, but is probably only effective over a long-term period. Among a narrow range of crops, we found the *Brassicacea* fodder radish showed substantial Zn and Cu removals, whereas management of the most toxic metals, such as As and Pb, still remains problematic. The much larger variability in shoot metal concentrations of the crops tested here compared with woody species suggests exploiting the potential of other herbaceous species, although we believe that more profitable progress could be achieved with an integrated approach involving genetics, biology, physiology and especially agronomy, to maximise plant adaptation and growth. In any case, identification of a pool of plants to be cultivated in association or in rotation is necessary, in order to cover the soil permanently and reduce possible damage by parasites. Assisted phytoextraction seems difficult to manage as regards timing and dosages of the compounds used and frequently reduce biomass yield and metal removal.

The phytomanagement of sites polluted by pyrite waste may simply involve the establishment of a vegetation cover with cultivated plants left to reproduce themselves or with biomass harvesting and annual sowing. However, besides phytoextraction, long-term stabilisation of metals in plant roots is an important issue to consider, in view of the high metal retention at root level, and recent evaluation at the University of Padova showed that 6 % of tap root biomass in rapeseed was recalcitrant to degradation after about 18 months from shoot harvest.

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Chapter 9
Phyto-transport and Assimilation of Selenium

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9.1 Selenium in the Environment

Selenium (Se) is one of the most widely distributed elements in the earth's crust and geographic distribution of Se in the environment is extremely variable (Hansen et al. 1998). In China, the average abundance of Se in the earth's crust is 0.058 mg kg^{-1}, which is slightly lower than that in other parts of the world (Xia and Tang 1990). While there are natural sources of Se, anthropogenic inputs of Se-containing chemicals into the environment are greater in amounts than natural contribution (Wu 2004). This has resulted in the elevated Se levels in natural ecosystems impacted by anthropogenic processes. Therefore, the risks derived from industrial activities and discharges have drawn widespread concern worldwide. Indeed, ample evidence showed that the increasing levels of Se have caused soil, air, and water pollution as well as changes in the structures of natural communities and ecosystems at organism levels (Ohlendorf et al. 1986; Banuelos et al. 1996).

In nature, Se occurs in four oxidation states with the chemical forms of selenide (Se^{2-}), elemental or "colloidal" Se (Se^{0}), selenite (Se^{4+}), and selenate (Se^{6+}) (Rosenfeld and Beath 1964). The most common and soluble species are selenate and selenite found mostly in seleniferous soils and agricultural drainage water (Banuelos and Lin 2005), whereas elemental Se dominates in anaerobic environment (Terry et al. 2000). Selenide is the ionized form of hydrogen selenide (H_2Se) and unstable in aqueous solutions (Barceloux 1999). Both selenate and selenite

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are water soluble, and therefore they possess greater bioavailability compared with other non-water soluble species of Se (Terry et al. 1992; Zhang and Moore 1997; Barceloux 1999; Shardendu et al. 2003; Banuelos and Lin 2005; Banuelos et al. 2005).

9.1.1 Anthropogenic Inputs of Selenium

One of the primary anthropogenic activities responsible for mobilizing Se in the ecosystem is the waste materials generated due to burning of fossil fuel like coal and petroleum oil during electric power production (Lemly 1985), which is almost 1,250 times higher than that in raw coal (Pillay et al. 1963). Due to the growing consumption for energy, more Se has been released or produced from the power industry globally. It is estimated that more than 1,600 tons of Se has been produced annually from mining production (Newland 1982; Nriagu and Pacyna 1988), accounting for 80 % of the total Se produced (Haygarth 1994). Se is also produced by oil refinery industry. Cure oil contains significantly higher concentrations of Se than coal, and procurement and refinery of oil produce huge volume of Se-laden wastes (Ohlendorf and Gala 2000; Simmons and Wallschläger 2005).

Selenium is widely used for a range of commercial products like glass and ceramics. Apart from huge utility in glass industry (approximately 20 % of its overall industrial use) (Newland 1982; Haygarth 1994), Se is also used in industries related to photoelectric cells, pigments, rectifiers, semiconductors, steel, and chemicals for photography and rubber vulcanizing (Barceloux 1999; Haygarth 1994). Additionally, Se is used in pharmaceutical industry for treating dandruff and fungal infection (Haygarth 1994).

9.1.2 Fate and Transport of Selenium in the Environment

The environmental fate and transport of Se depend on, in part, the rates and intermediates of the dynamic interconversion among the Se family, and in part the physical transfer of Se among the different environmental compartments (Wang and Gao 2001). It is known that there are substantial differences in the concentration, rates, extent, and speciation of Se in various environmental media (Porcella et al. 1991). Four different organic volatile forms of Se have been detected in air, namely methaneselenol (CH_3SeH), dimethyl selenide (CH_3SeCH_3), dimethyl selenenyl sulfide (CH_3SeSCH_3), and dimethyl diselenide ($CH_3SeSeCH_3$) (Chasteen 1998). Dimethylselenide (DMSe) is the most significant contributor to environmental Se mobility through air (Karlson et al. 1994). Other inorganic atmospheric Se species, such as hydrogen selenide (H_2Se), elemental Se, and selenium dioxide (SeO_2), can also be identified (Wen and Carignan 2007). Because Se can be released from various sources, speciation of atmospheric Se is highly variable and unstable.

Fate and transport of Se in water are strongly influenced by various environmental factors (Bowie and Grieb 1991). Selenate and selenite are two predominant chemical species in water, where the former one is more stable under alkaline and oxidizing conditions and the latter one is a dominant species in the mildly reducing environment (Barceloux 1999; Belzile et al. 2000). Uptake of Se by various organisms is able to immobilize Se temporarily (Simmons and Wallschläger 2005). Adsorption to clay, minerals, and dissolved organic carbon is also a process that immobilizes/sequesters Se in aquatic environment (Belzile et al. 2000). Additionally, chemical reduction of oxidized forms of Se to elemental/colloidal Se can be identified in water (Schlekat et al. 2000). It has been proposed that humic acids are the main reservoir of Se in soils (Tokunaga et al. 1991). Indeed, majority of Se in soils was detected in organic forms, namely salts of selenic acids and of selenious acids (Barceloux 1999). Insoluble species of Se, e.g., elemental Se, selenide, and selenium sulfides can also be identified in soils (Wang and Peng 1991). The elemental Se in kerogen is more steadily mobilized and accumulated by vegetation, whereas organically bound Se seems more resistant to chemical alteration and less bioavailable (Wen et al. 2006). Abundant literatures show that selenate and selenite rather than other Se species are easily taken up by plants (Terry et al. 2000; Zhang and Moore 1997; Shardendu et al. 2003). Microorganisms are able to methylate/convert elemental Se and selenite into volatile Se, DMSe, and dimethyl diselenide (DMDSe) (Doran 1982).

9.2 Toxicity of Selenium

Marco Polo probably recorded the first observations of Se toxicity to horses in western China in the thirteenth century (Dickerson and Smith 1994). Due to the narrow margin of Se concentration among its essentiality, deficiency, and toxicity, living organisms vary considerably in their physiological responses and tolerance to Se (Barceloux 1999; Hamilton 2004). Gaseous Se of DMSe produced by plants is 500–700 less toxic than selenate or selenite, with a lethal dose (LD_{50}) value of 1.6–2.2 g Se kg^{-1} for rat (Wilber 1980). An acceptable intake level has been documented at 3.5 mg L^{-1} by the USEPA (Atkinson et al. 1990). Whole-body Se threshold concentrations are suggested to be 6.0 and 9.0 mg kg^{-1} for cold water fish and warm water fish, while the dietary threshold at 10 and 11 mg kg^{-1} has been proposed, respectively (Hamilton 2003). The median LD_{50} of 8.8 mg Se kg^{-1} has been determined for selenomethionine (SeMet) (Ammar and Couri 1981) and selenite is fourfold more toxic than SeMet (Reid et al. 2004). The most common symptoms of Se poisoning are loss of hair and nails or lesion of skin, but nervous system and teeth may be affected in areas of higher incidence (Yang et al. 1983). The intravenous administration of Se compounds in mice resulted predominantly in cardiorespiratory effects, hind limb paralysis, and death (Ammar and Couri 1981). Growth inhibition, due to unacceptable high concentration of Se, has been often observed in microorganisms, aquatic plants, and animals (Simmons and Wallschläger 2005).

9.2.1 Selenium Essentiality

Selenium can be either beneficial or toxic to living organisms, highly depending on its chemical forms and the dose as well as other environmental regulating variables (Shardendu et al. 2003). The nutritional benefit of Se for preventing hepatic necrosis caused by vitamin E deficiency has been reported (Schwarz and Foltz 1957). Se is an essential constituent of the enzyme glutathione peroxidase (GSH-Px), which is an essential antioxidative system (Rotruck et al. 1973). GSH-Px assists in intracellular defense mechanisms against oxidative damage by preventing and reducing the production of reactive oxygen species (ROS) (Ursini and Bindoli 1987). More than 10 Se-containing proteins have been identified/or isolated, indicating that Se is not merely restricted to its role in antioxidant activity but also involved in other multiple aspects of mammalian metabolism (Tinggi 2003). The most famous case associated with Se deficiency is Keshan disease occurred in young Chinese children and women of childbearing age living in the low Se-belt regions in China (Wang and Gao 2001). It has been proposed that Se deficiencies in animals can be observed when diets contain less than 0.05–0.10 mg Se kg^{-1} (Milne 1998), or blood Se concentration is below 0.05 μg L^{-1} usually associated with clinical disease for people (Mass 1998). In spite of its nutrition and benefit to animals and humans, the question on the essentiality of Se as a micronutrient in higher plants is unresolved and remains controversial (Terry et al. 2000).

9.2.2 Mechanisms of Selenium Toxicity

Selenium supplementation with nutrient levels is able to increase GSH-Px activities, which is responsible for scavenging free radicals and neutralizing their potential damage (Hartikainen et al. 2000). However, oversupply of Se above the threshold of nutrition limit, in turn, increases oxidative stress and contributes to the formation of ROS (Seko and Imura 1997). ROS are generated as intermediates of a number of metabolic reactions in cellular organelles of different living organisms (Kitahara et al. 1993; Spallholz 1994). Inorganic Se compounds stimulate the formation of ROS, either by direct electron transfer involving cationic metals or as a consequence of metal-mediated inhibition of metabolic reactions (Halliwell and Gutteridge 1999). ROS can result in the damage of DNA, proteins, and pigments as well as initiating lipid peroxidation (Panda and Khan 2003). Adequate defense against oxygen toxicity requires efficient scavenging of ROS, e.g., superoxide radicals and hydrogen peroxide (Tsang et al. 1991). Superoxide radicals are toxic by-products of oxidative metabolism (Fridovich 1978). Toxicity of superoxide radicals has been attributed to its interaction with hydrogen peroxide to form highly reactive hydroxyl radicals, which are thought to be largely responsible for mediating oxygen toxicity in vivo (Fridovich 1978). The toxicity of Se at higher concentrations is thought to be mainly due to its chemical similarity to sulfur (S),

leading to nonspecific replacement of S by Se in proteins and other sulfur compounds (Pilon-Smits and LeDuc 2009). Additionally, a nonspecific integration of the selenoamino acids, selenocysteine (SeCys) and selenomethionine (SeMet), into proteins has been proposed to be the major contributor of Se toxicity in plants (Brown and Shrift 1982).

9.3 Remediation of Selenium

Various processes, ranging from intensive engineering techniques to bio-treatments, have been developed to remediate Se-contaminated soils and waters (Zayed et al. 1998). The majority of processes used for cleaning Se-contaminated wastewater are through physiochemical methods, such as chemical precipitation, electrochemical treatment, and catalytic reductions (Zayed et al. 1998). The use of ion exchange resins has primarily been used for the removal of soluble Se (Kashiwa et al. 2000). Aluminum oxide, manganese nodules, titanium oxide, hematite, and magnetite are adsorbents used for removing Se (EI-Shafey 2007). Bioremediation is a biological response to environmental abuse when the concentrations of the pollutants are below the threshold of toxicity to the exposed organisms. A broad range of bacteria, fungi, and yeasts have been identified or isolated to be capable of converting soluble Se into elemental Se and DMSe (Milne 1998). Because of the insolubility of elemental Se in aquatic systems, reduction of soluble Se to elemental Se is considered to be a useful technique for removing Se from Se-contaminated water (Zhang and Frankenberger 2005). In spite of the capabilities of microorganisms to reduce both Se chemical species, one conclusive result is that reduction of selenate by microorganisms is a more difficult process than selenite (Maiers et al. 1988). Indeed, selenate is often considered a spectator in living organisms (Milne 1998). Additionally, an algal–bacterial removal system has been designed, in which microalgae produced by the system provide sources of carbon and energy for the specific bacterial reduction of the soluble Se from wastewater (Lundquist et al. 1994).

9.4 Uptake and Transport of Selenium by Plants

The first interaction between Se and plants is during its uptake process. In spite of the capabilities of plants to take up different species of Se readily from soil solution, namely selenate, selenite, and organic Se compounds, great differences in the uptake and transport pathways between these Se species have been observed (Brown and Shrift 1982; Arvy 1993; White et al. 2004; Sors et al. 2005). Indeed, selenate is mainly absorbed by roots through cell membranes by sulfate transporters owing to the chemical similarity between selenate and sulfate (Brown and Shrift 1982; Smith et al. 1995). But very little is known about the mechanism of selenite

uptake by plant roots (Zhang et al. 2010). However, one conclusive result has been proposed in that selenite uptake may not be mediated by membrane transporters and it seems to be accumulated through passive diffusion process, whereas organic Se compounds' absorption by plants from the soil solutions is chiefly achieved by active processes (Arvy 1993; Sors et al. 2005). Uptake and transport of Se by various plants are well documented. Plants of the genus *Astragalus*, *Neptunia*, *Stanleya*, *Morinda*, *Oonopsis*, *and Xylorhiza* have been found to be able to hyperaccumulate Se in their shoots while they grow normally on soils with natural Se (Virupaksha and Shrift 1965; Brown and Shrift 1982; Davis 1986). In contrast, Se non-hyperaccumulators do not accumulate Se above 100 mg Se kg^{-1} DW when grown on seleniferous soils (Brown and Shrift 1982; Terry et al. 2000).

9.4.1 Factors Affecting Uptake and Transport

The rate of botanical uptake and transport of Se depends on the concentrations and chemical forms of Se in the soil, ionic forms in the solution, as well as rhizosphere conditions such as pH and redox potential, and the presence of sulfate and phosphate, which compete with Se uptake (Bell et al. 1992; Blaylock and James 1994; Dhillon and Dhillon 2003; Sors et al. 2005; Sun et al. 2010). Selenate is the predominant form of Se in alkaline and well-oxidized soils ($pe + pH > 15$), whereas in well-drained mineral soils with pH from acidic to neutral ($7.5 < pe + pH < 15$), Se exists predominantly as selenite (Elrashidi et al. 1987; Zhu et al. 2009). Under strongly reduced soil conditions ($pe + pH < 7.5$), selenide becomes the dominant form (Elrashidi et al. 1987; Zhu et al. 2009). The dependency of Se bioavailability on redox condition, pH, and competing ions is complicated by the dynamic environment present in the rhizosphere, when plant roots and microorganisms can change the conditions over time (Blaylock and James 1994; Zhang et al. 2010).

Selenite and selenate are the two most common chemical forms in the family of Se in the environment. The former has a strong affinity of sorption, while the latter is more water soluble (Hamilton 2003) and both can be easily taken up by plants (Terry et al. 1992; Zhang and Moore 1997; Shardendu et al. 2003; Banuelos and Lin 2005; Banuelos et al. 2005; Yu and Gu 2007; Sun et al. 2010; Freeman and Banuelos 2011; Quinn et al. 2011). Much more and faster Se removed can be observed when detached roots of willows are exposed to selenate than to selenite (De Souza et al. 1998; Zhao et al. 2005; Yu and Gu 2008), suggesting that independent botanical uptake pathways exist in plants between the two Se species. Additional efforts from greenhouse experiments show that the uptake rate of selenate by willow cuttings was approximately 2.86-fold higher than that of selenite (Yu and Gu 2007). A higher result has also been reported by Zayed et al. (1998), in which the total Se uptake was greater in selenate-supplied plants (4–5-fold higher) than in selenite-supplied ones. One interesting result showed that detached leaves of willows have been found to be unable to take up either selenate or selenite from the

hydroponic solution (Yu and Gu 2008), implying that both Se species are unable to pass through the cuticle of the leaves, which are the limiting barriers in foliar uptake of a wide range of chemicals (Schönherr and Riederer 1989).

Competitive inhibition of selenate by sulfate is well documented and can be ascribed to the chemical similarity of the two ions (Bell et al. 1992). Both selenate and sulfate are transported across the plasma membrane of root epidermal cells against their electrochemical gradients, with uptake being driven by the cotransport of three protons for each ion (Lass and Ullrich-Eberius 1984; Hawkesford et al. 1993; Sors et al. 2005). It is known that *Arabidopsis thaliana* mutants that lack a functional sulfate transporter are resistant to selenate (Shibagaki et al. 2002; Yoshimoto et al. 2002; Whanger 2002; Ellis and Salt 2003). Increasing sulfate supply in the plant growth medium resulted in a progressive inhibition in selenate uptake, but it caused little or no effect on selenite and SeMet uptake (Zayed et al. 1998). Phosphate is not expected to be particularly inhibitory for selenate uptake because of their chemical dissimilarities (Hopper and Parker 1999). However, uptake of selenate by alfalfa was decreased by increasing phosphate from 32 to 129 mM (Khattak et al. 1991). The ability of phosphate to inhibit selenite uptake by plants is also apparent; however, competitive inhibition of selenite uptake by phosphate occurs across diverse plant genotypes (Hopper and Parker 1999). Indeed, increasing phosphate caused a decrease by 30–50 % in Se content of ryegrass shoots and roots exposed to selenite, while only the roots of strawberry and clover showed comparable inhibition of selenite uptake (Hopper and Parker 1999). Another interesting conclusion is that the inhibition of selenite uptake in non-accumulating species is somewhat stronger than that in accumulating species (Broyer et al. 1972; Hopper and Parker 1999).

It is known that the capability of plants to accumulate Se in their tissues highly relies on their genetic traits. There are significant differences in the degree of tolerance, uptake, and accumulation of Se among different species of plants (Wu et al. 2003; Srivastava et al. 2005; Banuelos et al. 2005). According to Se bioaccumulation capacity, plants can be divided into three groups: primary accumulators (hyperaccumulators), secondary accumulators, and non-accumulators (Dhillon and Dhillon 2003; White et al. 2007). A limited number of plants, especially from the family of *Fabaceae* and *Brassicaceae*, are able to accumulate considerably higher levels of Se in plant materials, when grown on seleniferous soils (Pilon-Smits et al. 1999; White et al. 2004; De Fillips 2010). The ability of Se hyperaccumulator plants to accumulate and tolerate high concentrations of Se is thought to be associated with a distinct metabolic capacity that enables them to divert Se away from incorporation into proteins (Brown and Shrift 1982; Pilon-Smits and LeDuc 2009). Translocation of Se to the shoots from the roots is largely dependent on the form of Se supplied. Completely different results have been reported by different research groups. For examples, ryegrass translocation percentages (percentage of total Se taken up located in shoots at harvest) ranged from 84 to 91 % for selenate and from 44 to 46 % for selenite (Hopper and Parker 1999). Selenate was rapidly translocated to the shoots in Indian mustard, away from the roots, whereas approximately 10 % of the selenite was translocated (De Souza

et al. 1998). The shoot/root ratio of total Se content in plants ranged from 0.6 to 1 for plants supplied with SeMet and was less than 0.5 for those supplied with selenite, while this ratio can range from 1.4 to 17.2 when selenate is the only form of Se supplied (Zayed et al. 1998). On the contrary, selenite is more mobile than selenate after uptake by plant roots, although more selenate was eliminated by willows from the plant growth medium than selenite (Yu and Gu 2008). Indeed, the translocation efficiency of selenite was more than onefold higher than that of selenate (Yu and Gu 2008).

9.4.2 Selenium Assimilation and Metabolism

The chemical and physical resemblance between Se and S establishes that both elements share common metabolic pathways in plants (Sors et al. 2005). It has been proposed that selenate is primarily transported into the chloroplasts, where it is metabolized by enzymes of S assimilation (Leustek et al. 2000; Ellis and Salt 2003). The same Se species was only detected in the roots of de-topped plants supplied with selenate, supporting that the chloroplasts are the sites for ATP sulfurylase activity and selenate reduction (Pilon-Smits et al. 1999; De Fillips 2010). In vitro ATP sulfurylase has been shown to be able to activate sulfate (Leustek et al. 1994), while the reduction of selenate to adenosine phosphoselenate (APSe) catalyzed by ATP sulfurylase is also suggested (Fig. 9.1) (Leustek et al. 1994; Sors et al. 2005). Indeed, overexpression of ATP sulfurylase in Indian mustard has confirmed that the activation of selenate to APSe by ATP sulfurylase is one of the rate-limiting steps for selenate assimilation in plants (Pilon-Smits et al. 1999; Ellis and Salt 2003). Evidence is also available on that bound APSe can be further assimilated and/or metabolized via either non-enzymatically or enzymatically pathways (Ng and Anderson 1979; Terry et al. 2000). During the enzymatically catalyzed pathway, APSe is converted into selenite by adenosine 5-phosphosulfate (APS) reductase (Terry et al. 2000). Due to the possibility of the conversion of selenate into selenite in the biosynthesis of organic Se compounds (De Souza et al. 1998), selenite is able to subsequently non-enzymatically reduce into selenide in the presence of glutathione in vitro (Ng and Anderson 1979). Therefore, we have a good reason to propose that the existence of this nonenzymatic pathway for the reduction of selenite to selenide explains why selenite is more readily assimilated by plants than selenate (De Souza et al. 1998; Ellis and Salt 2003).

The sequential process is the production of SeCys due to the selenide assimilation (Terry et al. 2000), in which SeCys is formed by the action of cysteine (Cys) synthase, which couples selenide with O-acetylserine (Ng and Anderson 1978). Ultimately, SeCys can enter the methionine (Met) biosynthetic pathway via selenocystathionine (SeCysth) and selenohomocysteine (SeHoCys) to form SeMet (Sors et al. 2005). Both SeCys and SeMet are nonspecifically incorporated into proteins, which contribute to the phytotoxicity of Se (Brown and Shrift 1982; EI Mehdawi and Pilon-Smits 2011). Clearly, the spinach cystathionine-γ-synthase

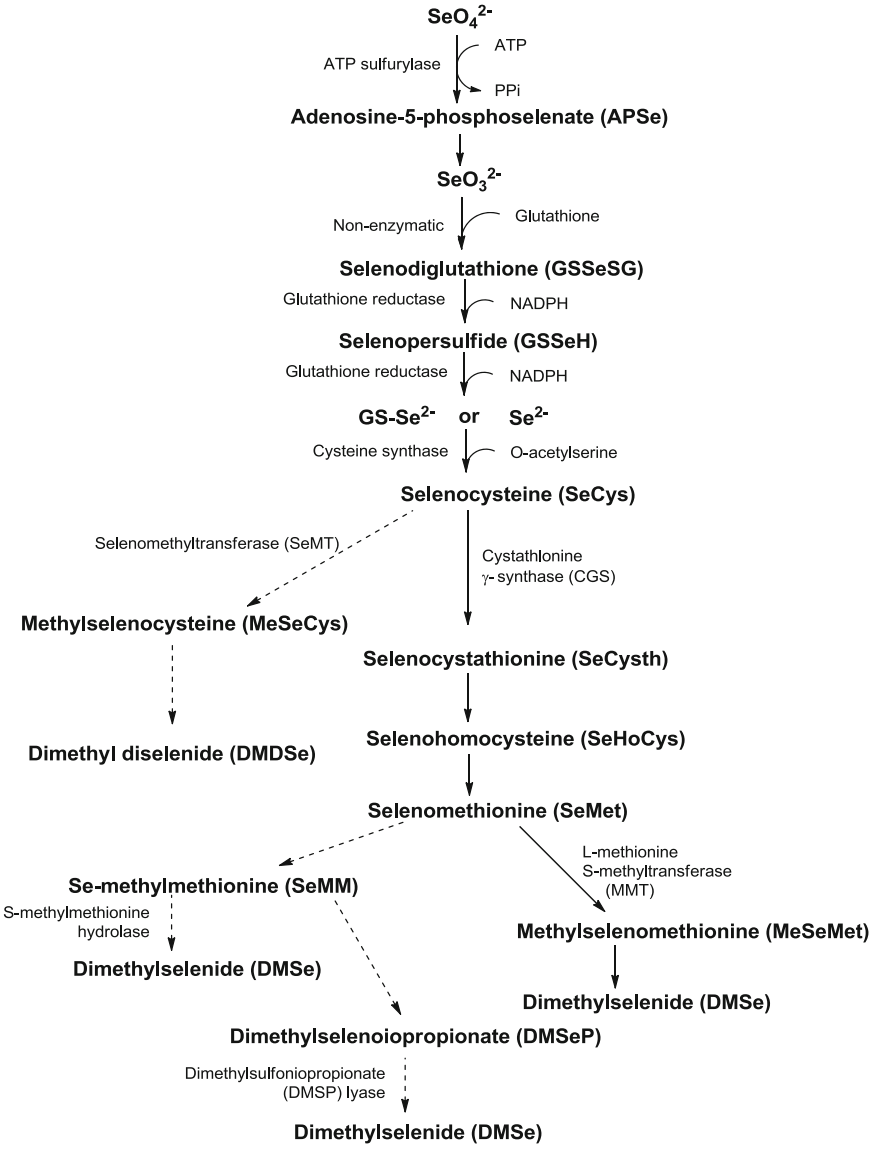

Fig. 9.1 Generalized Se metabolism in plants adapted from Parker et al. (2003) with some modification

(CGS), which is responsible for catalyzing the formation of SeCysth, displays greater affinity for SeCys (K_m = 70 μM) than Cys (K_m = 240 μM) (Dawson and Anderson 1988). However, the enzymatic bioassays of CGS from Se hyperaccumulating and non-accumulating *Astragalus* species revealed that the enzyme from both groups had similar properties, suggesting that the Se accumulation and

tolerance traits in *Astragalus* are not related to CGS kinetics (Dawson and Anderson 1988; Sors et al. 2005).

SeMet synthesized by the Met biosynthetic pathway can be methylated and converted into DMSe, which is the major species of volatile Se compounds produced (Tagmount et al. 2002). The first committed step involved in the production of DMSe is the methylation of SeMet to form methylselenomethionine (MeSeMet), which is catalyzed by the enzyme *S*-adenosyl-L-methionine: L-methionine *S*-methyltransferase (MMT) (Tagmount et al. 2002; Ellis and Salt 2003; Sors et al. 2005). More than likely, the botanical conversion of Se-methylmethionine (SeMM) to DMSe is catalyzed by S-methylmethionine hydrolase (Ellis and Salt 2003; Sors et al. 2005), which is widely observed in plants during the conversion of S-methylmethionine (SMM) to dimethylsulfide (DMS) (Pimenta et al. 1998). Another possible biochemical pathway is also suggested, in which DMSe is likely produced by the conversion of SeMM to the intermediate dimethylselenoiopropionate (DMSeP) in the chloroplast (De Souza et al. 1998). Indeed, Indian mustards supplied with DMSeP are able to volatilize significantly higher Se than plants supplied with SeMet (De Souza et al. 1998; Sors et al. 2005). It has been suggested that dimethylsulfoniopropionate (DMSP) lyase is responsible for the conversion of DMSeP to DMSe; however, DMSP lyase has not yet been identified in plants (De Souza et al. 1998; Ellis and Salt 2003). Due to the leaf portion being exposed to the air, plants are able to transpire the resultant methylated forms of Se into the atmosphere through leaves. Actually, Se volatilization is the process by which gaseous forms such as DMSe and DMDSe are produced from other inorganic or organic forms of Se (Terry et al. 1992; Zayed et al. 1998; De Souza et al. 1998; Meija et al. 2002; Yu and Gu 2008). The rate of Se volatilization varies with plants species. Willow cuttings likely transpired approximately 10 % applied Se in forms of selenate and selenite (Yu and Gu 2008). As much as 10–30 % of the Se can be removed by biological volatilization (Hansen et al. 1998), whereas wetland plants showed a 50-fold variation in Se volatilization (Duckart et al. 1992). Additionally, plants supplied with selenite volatilized more Se than selenate (Zayed et al. 1998). Because DMSe is less toxic than other species of Se (De Souza et al. 1998), phyto-volatilization has drawn more attention as a possible method for the phytoremediation of Se-contaminated soils (Terry et al. 1992).

9.4.3 Genetics Involved in Selenium Metabolism in Plants

Despite the existence of naturally occurring Se-accumulating plants, an interest has been generated in using unusual plants as tools to improve and clarify our basic understanding of Se biochemistry in plants (Ellis and Salt 2003). Indeed, different genes related to Se tolerance, accumulation, and metabolism have been identified or isolated. For example, overexpression of the *Arabidopsis* APS1 genes encoding a plastidic ATP sulfurylase in Indian mustard has been found to be able to increase the assimilation of selenate into SeMet, whereas the wild-type accumulated Se in

plant materials as selenate (Pilon-Smits et al. 1999). Se tolerance and accumulation in APS transgenic plants was also significantly higher than untransformed plants (Pilon-Smits et al. 1999). Similarly, the APS transgenics are able to contain 2.5-fold higher shoot Se levels compared with the wild type of Indian mustards (Van Huysen et al. 2004). However, there is no difference in cell growth or sensitivity to selenate between transgenic (overexpression of APS2 genes encoding ATP sulfurylase) and wild-type cells of tobacco (Hatzfeld et al. 1998). CGS mediates the conversion of SeCysth from SeCys. Transgenic Indian mustards that overexpress CGS were shown to have 2–3-fold higher Se volatilization rate, 20–40 % lower shoot Se level and 50–70 % root Se levels, and higher Se tolerance than the wild type (Van Huysen et al. 2003). The higher Se volatilization rates of the CGS transgenics suggest that CGS is rate limiting for Se volatilization as DMSe (Van Huysen et al. 2004).

In Se hyperaccumulating plants, the amino acids methylselenocysteine (MeSeCys) and methylcysteine (MeCys) are produced from the methylation of SeCys and Cys in the presence of the enzyme selenomethyltransferase (SeMT) using SMM as the methyl donor (Neuhier et al. 1999; Ellis and Salt 2003). Indeed, Indian mustard transgenic plants accumulated more Se in the form of MeSeCys than the wild type, using SeMT gene probe from Se hyperaccumulator *Arabidopsis* (Leduc et al. 2004). Additionally, SeMT transgenic seedlings tolerated Se (particularly selenite) better than untransformed plants, producing 3–7-fold greater biomass and 3-fold longer root lengths (Leduc et al. 2004). A similar finding has been reached that MeSeCys accumulation in transgenic broccoli closely correlated to the SeMT gene expression (Lyi et al. 2005). Obviously, Se accumulation in genetically engineered plants provided important information for maximizing MeSeCys production in beneficial vegetable plants (Leduc et al. 2004; Lyi et al. 2005).

It is noted that overexpression of SeMT in plants would be expected to lead to increased methylation of SeCys, resulting in decreasing production of SeMet (Ellis and Salt 2003). Consequently, the resulting reduction in SeMet would decrease the formation of DMse. However, SeMT overexpressing Indian mustards has significantly increased Se volatilization compared with the wild type (Leduc et al. 2004). This is because a different assimilation pathway exists in plants, in which the MeSeCys produced from the methylation of SeCys is further converted into another volatile species DMDSe rather than DMSe (Meija et al. 2002).

Other genes have also been isolated. The APS reductase (PaAPR) was isolated from the bacterium *P. aeruginosa* and expressed in *A. thaliana* (Bruhl et al. 1996). Plants supplied with selenate increase Se reduction by 50–80 %, suggesting the capacity of reducing APSe (Bruhl et al. 1996; De Fillips 2010). SeCys lyase is the enzyme involved in Se assimilation. In transgenic *B. juncea* originally sourced from *A. thaliana*, overexpression of SeCys lyase is able to reduced selenate toxicity (Banuelos et al. 2007), which attributes to a reduction in the incorporation of Se into proteins. Banuelos et al. (2007) also cloned and expressed the gene of SeCys transferase, which has little effect on selenate toxicity, but causes a small effect on selenite toxicity.

9.5 Conclusions

The extensive use of Se-containing chemicals due to anthropogenic activities has resulted in significant releases and its distribution in the environment. Speciation of Se in different environmental compartments is substantially different. Among the chemical form of Se, the most common species are selenate and selenite, which display quite different chemical properties. The former is more water soluble, while the latter has a strong affinity of sorption. Both Se species are bioavailable for plants. Selenate is chiefly absorbed by roots through cell membranes by active transport driven by ATP (ATPase), whereas selenite uptake may not be mediated by membrane transporters and seems to be accumulated through passive diffusion. Due to the chemical similarity between selenate and sulfate, both elements share the common metabolic pathway in plants. Competitive inhibition in biochemical processes between selenate and sulfate affects uptake, translocation, and assimilation throughout plant development. Through reviewing the Se uptake, transport, assimilation, and volatilization in plants, it is evident that the ability of plants to accumulate Se in their tissues highly relies on their genetic traits and greater differences in the capacities of tolerance, uptake, accumulation, assimilation, and volatilization of Se among various species of plants. The ATP sulfurylase pathway responsible for the botanical reduction of selenate has been widely observed in plants. Since this biological process is rate limiting enzymatically, storage of selenate in plant materials is more likely to be in selenate-supplied plants. Indeed, ATP transgenic Indian mustards are able to increase the assimilation of selenate into SeMet, whereas selenate accumulated in the wild-type plant supplied with selenate. Due to the existence of a nonenzymatic pathway capable of reducing selenite into selenide, selenite is more readily assimilated by plants than selenate. The major gaseous Se of DMSe has been identified, which is the most significant contributor to the environment Se mobility in atmosphere. Since the volatile DMSe is much less toxic than other species of Se, phyto-volatilization is a suggestive remediation strategy for phytoremediation of Se-contaminated soils. Although hyperaccumulators exhibit much more promise in the removal and accumulation of Se than non-accumulators, most hyperaccumulators belong to grasses, which may serve as food sources for numerous higher animals. However, Se hyperaccumulators are able to provide a source of genetic materials that can be used to modify or alter the botanical capacity of Se uptake, transport, and assimilation using molecular modification of genes encoding proteins. It is noted that the introduction of any transgenic plant into ecosystems should never be taken lightly; it needs to be accompanied by careful risk assessment since it is very difficult to identify beforehand the ecological consequences of releasing transgenics into the environment.

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Chapter 10
Phytostabilization as Soil Remediation Strategy

Agustina Branzini and Marta S. Zubillaga

10.1 Anthropogenic Pollution with Heavy Metals

Heavy metals are natural components of the Earth's crust, being ubiquitous in low amounts in terrestrial ecosystem. High natural levels of metals originating from geological processes are occasionally found, but in many terrestrial ecosystems the concentration of several heavy metals has reached toxic levels as a consequence of anthropogenic activities (Zhang et al. 2005). Fifty-three elements fall into the category of heavy metals to date, and heavy metals are defined as the group of elements whose densities are higher than 5 g cm^{-3} and are recognized as environmental contaminants in industrialized societies (Padmavathiamma and Li 2007).

Diffuse and point pollution of soils by heavy metals is a major environmental problem worldwide (Kumpiene et al. 2006). In particular, soils could become contaminated by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Khan et al. 2008; Zhang et al. 2010) (Fig. 10.1). One important effect of heavy metals in the soil from anthropogenic sources is that they tend to be more mobile and bioavailable than those from pedogenic or lithogenic ones (Kaasalainen and Yli-Halla 2003). Soil pollution caused by metals is somewhat different from air or water pollution, because heavy metals persist in soil much longer than in other compartments of the biosphere (Lasat 2002). In general, soil heavy metal contamination might pose direct or indirect risks to humans and the ecosystem through ingestion or contact with contaminated soil, the food chain (soil–plant–human or

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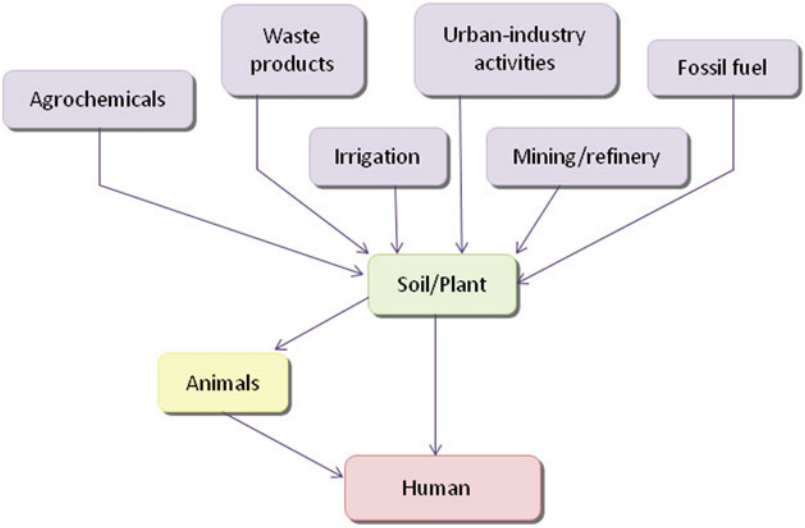

Fig. 10.1 Contamination sources of the soil–plant–animal system

soil–plant–animal–human), drinking of contaminated groundwater, reduction in food quality (safety and marketability) via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems (Ling et al. 2007). In fact, one of the most important problems of heavy metals' contamination, related with their nondegradable condition, is that their accumulation in the food chain will have a significant effect on human health in the long term (Gleyzes et al. 2001).

In an ecological research, any metal or metalloid that causes environmental problem, which cannot be biologically degraded, should be considered as a heavy metal. Therefore, heavy metals represent an ill-defined group of inorganic chemical hazards, and those most commonly found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (McLaughlin et al. 1999). Out of the 92 known elements present on the earth crust, some metals are well known to be essential micronutrients for plants and animals, and others have no known biological function.

Essential nutrients could be defined as those without which plants and animals cannot complete their life cycle, irreplaceable by other elements, and directly involved in plant/animal metabolism. Consequently, certain levels of micronutrients are necessary to mediate the numerous biochemical reactions essential for growth and development. Based on the quantity required, nutrients are divided into macro- and micronutrients. Micronutrients have also been called minor or trace elements, indicating that their concentrations in tissues are minor or in trace amounts relative to the macronutrients (Mortvedt 2000). For plants, recycling organic matter such as grass clippings and tree leaves is an excellent way of

providing micronutrients (as well as macronutrients) to growing plants. These essential micronutrients are boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn). The accumulation of these micronutrients by plants generally follows the order of Mn > Fe > Zn > B > Cu > Mo. This order may change among plant species and growth conditions. However, at elevated bioavailable concentrations in soil and when taken in excessive amounts, all metals ions may cause toxic effects on plants and animal organisms, including humans (Fig. 10.1) (Küpper and Kroneck 2005). In this chapter, the focus is on essential and nonessential elements that will be a risk for environment and humans and that result from industrial activities.

10.1.1 Copper

Copper is the third most used metal in the world (VCI 2011). Copper ranks 26th behind zinc in abundance on the lithosphere, and it is a naturally occurring element, which can be found in all environmental media: air, soil, sediment, and water (Alloway 1995). Concentrations of Cu in soils range from about 2 to 100 mg kg^{-1} with a mean of 30 mg kg^{-1} (Mortvedt 2000). Cu is mostly found in silt and clay fractions of soil and usually present in carbonate fractions in alkaline soils and in Fe oxide fractions in acid soils. Also, it occurs in numerous minerals including cuprite, tenorite, malachite, azurite, and native copper. Copper forms sulfides, sulfates, sulfosalts, carbonates, and other compounds and occurs in reducing environments as the native metal. In the soil, Cu strongly complexes to the organic implying that only a small fraction of copper will be found in solution as ionic copper, Cu(II). Cu is an essential micronutrient required in the growth of both plants and animals. In humans, it helps in the production of blood hemoglobin. In plants, Cu is especially important in seed production, disease resistance, and regulation of water. Copper is indeed essential, but in high doses it can cause anemia, liver and kidney damage, and stomach and intestinal irritation.

10.1.2 Zinc

Zinc is the second most abundantly distributed element in the body after iron. Zinc occurs naturally in soil (about 70 mg kg^{-1} in crustal rocks), but Zn concentrations are rising unnaturally, due to anthropogenic additions. Water-soluble Zn that is located in soils can contaminate groundwater. In effect, some fish can accumulate Zn in their bodies, and it is able to biomagnify up the food chain. Plants often have a Zn uptake that their systems cannot handle, due to the accumulation of Zn in soils. Zn catalyzes enzyme activity, contributes to protein structure, and regulates gene expression. Also, zinc is involved in the carbohydrate transformation (consumption of sugars) and in plant development regulation. Finally, Zn can interrupt the activity

in soils, as it negatively influences the activity of microorganisms and earthworms, thus retarding the breakdown of organic matter (Greany 2005). The Zn sources are soil, zinc oxide, zinc sulfate, and zinc chelate, and Zn^{2+} cation is the predominate form taken up by plants.

10.1.3 Cadmium

Cadmium compounds are, compared to other heavy metals, relatively water soluble. Therefore, these compounds are further mobile and available in soil and tend to bioaccumulate. The average natural abundance of Cd in the earth's crust has most often been reported from 0.1 to 0.5 ppm. In contaminated soils, Cd is derived from both natural and anthropogenic sources. Natural sources include underlying solid rock or transported parent material such as glacial till and alluvium. Anthropogenic input to soils occurs by aerial deposition and sewage sludge, manure, and phosphate fertilizer application. The major factors governing Cd 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 (Kim and Kim 2010). Its persistence in the environment and its relatively rapid uptake and accumulation by food chain crops are factors contributing to its potential environmental hazards. Cadmium concentrations of air ambient rarely exceed 0.01 g m^{-3}. However, cigarette smoking adds considerably to Cd input via inhalation. On the other hand, even though acute Cd toxicity caused by food consumption is rare, chronic exposure to high Cd levels in food can significantly increase the accumulation of Cd in certain body organs. Cd accumulates in the human body and especially in the kidneys. When Cd concentration in human body reaches levels considered to be harmful [$>$200 mg kg^{-1} wet weight in the kidney cortex according to Kjellstrom and Nordberg (1978)], this metal could induce kidney damage and led to its dysfunction with impaired reabsorption of, for instance, proteins, glucose, and amino acids.

10.1.4 Nickel

Nickel combined with other elements occurs naturally in the earth's crust. It is found in all soils, and is emitted from volcanoes. However, it normally occurs at very low levels in the environment, and it is primarily found combined with oxygen or sulfur as oxides or sulfides. Soil usually contains between 4 and 80 parts of nickel in a million parts of soil (ppm). The highest soil concentrations (up to 9,000 ppm) are found near industries that extract nickel from ore. Ni can also be released in industrial wastewater. As a result, a lot of Ni released into the environment ends up in soil or sediment where it strongly attaches to particles containing iron or manganese. Under acidic conditions, Ni is more mobile in soil and might seep

into groundwater. Studies show that some plants can take up and accumulate Ni. However, it has been shown that Ni does not accumulate in small animals living on land that has been treated with Ni-containing sludge. In humans, food is the major source of exposure to nickel. Also, the exposure to Ni may be breathing air, drinking water, or smoking tobacco containing Ni. The most common harmful health effect of nickel in humans is an allergic reaction. Approximately 10–20 % of the population is sensitive to nickel.

10.1.5 Lead

Lead is not essential for plant or animal life, and in the environment it is mainly particulate bound with relatively low mobility and bioavailability. Lead does, in general, not bioaccumulate and there is no increase in concentration of the metal in food chains. In humans, Pb can result in a wide range of biological effects depending upon the level and duration of exposure. For infants and young children Pb in dust and soil often constitutes a major exposure pathway and this exposure has been one of the main concerns as to the exposure of the general population. Absorbed Pb is rapidly taken up into blood and soft tissue, followed by a slower redistribution to bone. Bone accumulates Pb during much of the human life span and may serve as an endogenous source of Pb that may be released slowly over many years after the exposure stops. In the environment Pb binds strongly to particles, such as soil, sediment, and sewage sludge. Because of the low solubility of most of its salts, Pb tends to precipitate out of complex solutions. Consequently, the fate of Pb in the soil is affected by the specific or exchange adsorption at mineral interfaces, the precipitation of sparingly soluble solid phases, and the formation of relatively stable organo-metal complexes or chelates with the organic matter in soil (Gustafsson et al. 2012). The tendency of inorganic Pb to form highly insoluble salts and complexes with various anions together with its tight binding to soils drastically reduces its availability to terrestrial plants via the roots. Lead is taken up by terrestrial plants through the roots and to a lesser extent through the shoots. Translocation of the ion in plants is limited and most bound Pb stays at root or leaf surfaces. As a result, in most experimental studies on lead toxicity, high lead concentrations in the range of 100–1,000 mg kg^{-1} soil are needed to cause visible toxic effects on photo synthesis, growth, or other parameters. Thus, Pb is only likely to affect plants at sites with very high environmental concentrations.

10.1.6 Chromium

Chromium is the 21st most common element in the earth's crust. Also, Cr is found in all phases of the environment, including air, water, and soil. Naturally, occurring in soil, Cr ranges from 10 to 50 mg kg^{-1} depending on the parental material. Cr and

its compounds have multifarious industrial uses. They are used in the electroplating industry as anticorrosive and antibiofouling agents, in steel production, automobile manufacturing, and catalytic manufacture, and in the production of chromic acid and specialty chemicals. These anthropogenic activities produce general Cr contamination in the environment and have increased its bioavailability and mobility (Shanker et al. 2005). Among the factors that affect the Cr speciation in soil and water and its uptake into animals and plants include organic matter content, ferrous ion content, redox state, and pH (Kotas and Stasicka 2000). However, Cr is in general not bioaccumulated and there is no increase in concentration of the metal in food chains. In the natural environment, chromium occurs as two oxidation states or valences: chromium (III) and chromium (VI). The stable forms of Cr are the trivalent Cr(III) and the hexavalent Cr(IV) species, although there are various other valence states which are unstable and short-lived in biological systems. There is a great difference between Cr(III) and Cr(VI) with respect to toxicological and environmental properties, and they must always be considered separately. Cr(III) is less mobile, less toxic than Cr(VI), and is mainly found bound to organic matter in soil (Becquer et al. 2003). In general, chromium (VI) is favored by higher pH, aerobic conditions, low amounts of organic matter, and the presence of manganese and iron oxides which oxidize chromium (III). Cr(III) is an essential nutrient for animals and humans in amounts of 50–200 μg per day, being necessary for the metabolism of insulin. On the other hand, Cr(VI) has been demonstrated to have a number of adverse effects ranging from causing irritation to cancer. Effects in humans occupationally exposed to high levels of chromium or its compounds, primarily Cr(VI) by inhalation, may include irritating respiratory effects, possible circulatory effects, effects on stomach and blood, liver and kidney effects, and increased risk of death from lung cancer. Although Cr is present in all plants, it has not been proved to be an essential element for plants. Several factors affect the availability of Cr for the plant, including the soil pH, interactions with other minerals or organic chelating compounds, and carbon dioxide and oxygen concentrations. Little Cr is translocated from the site of absorption; however, the chelated form is transported throughout the plant. Chromium in high concentrations can be toxic for plants, and the main feature of Cr intoxication is chlorosis, which is similar to iron deficiency.

10.1.7 Mercury

Mercury is a peculiar metal. Most conspicuous is its fluidity at room temperature, but more important for the possible exposure of humans and the environment to mercury are two other properties:

- Under reducing conditions in the environment, ionic mercury changes to the uncharged elemental mercury, which is volatile and may be transported over long distances by air.

- Mercury may be chemically or biologically transformed to methylmercury and dimethylmercury, of which the former is bioaccumulative and the latter is volatile and may be transported over long distances.

Mercury is not essential for plant or animal life. The main human exposure to Hg is via inhalation of the vapor of elemental Hg and ingestion of methylmercury compounds in food. This compound affects among other organs also the brain, and it is documented that (as for lead) children in the embryonic stage receive mercury via the placenta causing persistent effects on children's mental development. However, the Hg toxicity varies among the different types of Hg. Generally, organic forms are much more toxic than the inorganic forms.

10.1.8 Arsenic

Arsenic is a silver-gray or white metallic solid element found in nature. Arsenic combines with other elements to form organic and inorganic compounds, inorganic arsenic compounds being more toxic than organic arsenic compounds. Soils and waters containing high levels of arsenic compounds can easily contaminate plants, animals, and human beings in contact with them, as they either produce toxic effects or accumulate in plants and thereby enter animal and human food chains (Nriagu 1994). Several thousand people consuming untreated groundwater might have a considerable health risk and there would be a harmful influence on the development of agriculture and cattle raising activities. The disease ascribed to arsenic contamination was later called 'chronic endemic regional hydroarsenism' (HACRE, 'hidroarsenicismo crónico regional endémico', in Spanish).

10.2 Heavy Metals in Environment

Heavy metals that are introduced into soils accumulate mainly in the upper layers of soils (Smith 1996). In general, this accumulation allows plants to uptake them, and through a biomagnification process in the food chain, they can constitute a serious health problem for animals and humans. Nevertheless, their mobility in soil could be reduced due to high capacity of soil material to adsorb heavy metals. When heavy metals are added to soils, some of them may chemically or physically interact with the natural compounds of soils, being immobilized or forming compounds that have low solubility. This degree of sorption is predominantly affected by environmental factors, soil components, and properties as well as the amount of heavy metals added (Jalali and Khanlari 2008). In fact, metal transport is dependent on the physiochemical properties and the amount of the metals, but mostly on the physical and chemical properties of the soil. As a result, the primary soil factors controlling the potential bioavailability of metals are soil organic matter content, soil pH, the

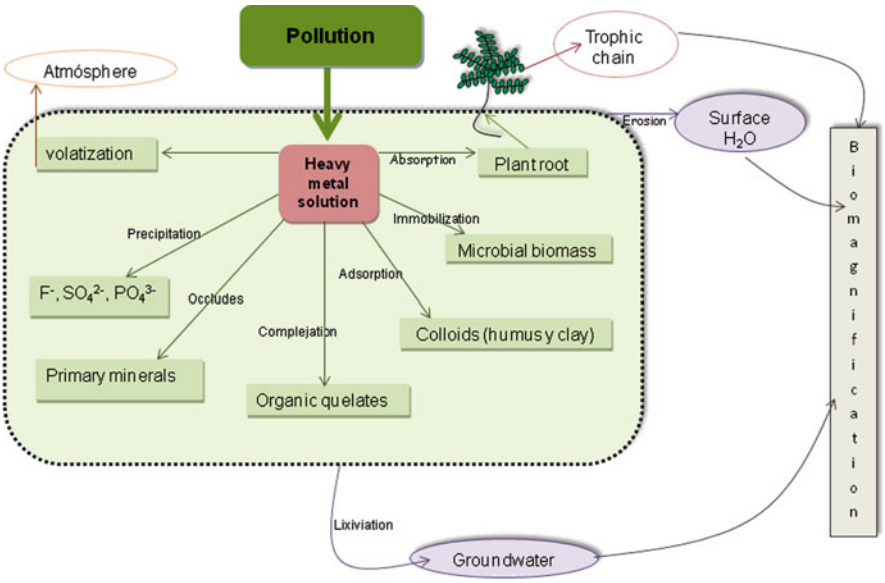

Fig. 10.2 Dynamics of heavy metals in soil. Adapted from Pierzynski et al. (2005)

accessibility and character of sorption sites on soil surfaces, the contents of Fe and Al oxyhydroxides, clay fraction content, and the cation exchange capacity (mineralogical composition) (Alvarez et al. 2008). However, according to USEPA (1993), the first two of these factors are the most important for controlling the release of metals to pore water and their subsequent bioavailability. It should be noted that the residence time of heavy metals in soil is directly related to their bioavailability. Also, as we see in Fig. 10.2, pollution of the environment by heavy metals poses a threat to surface water and groundwater, which are used as the main sources of drinking water by many inhabitants in the world.

On the other hand, metals present in soil often come in mixtures of three to five, depending on the source of contamination. That is why an increased concentration and simultaneous application of metals into soil creates increased competition between cations and metals for binding sites, thus controlling their solubility (Ghosh and Singh 2005). Several works on the biological effects of heavy metals have focused on the action of single contaminants against test organisms (Enserink et al. 1991; Parrott and Sprague 1993). However, these works have disregarded the fact that interactions can occur when two or more heavy metals are applied simultaneously to the environment and that their combined effect may result in an increase (synergism) or a decrease (antagonism) of the toxicity of the separate heavy metals (Otitoloju 2003). Also, since the heterogeneity of soils makes it very difficult to predict the potential mobility and distribution of single metals, experimental data are essential (Cerqueira et al. 2011). Mixture toxicity experiments may reflect the actual pollution of ecosystems in a more realistic way than experiments in which toxicants are tested individually (Spurgeon et al. 1994).

10.3 Remediation Strategies

Remediation is essential to mitigate the negative effects caused by the heavy metals incorporated to ecosystems, alone or in mixtures. The overall objective of any soil remediation approach is to create a final solution that protects human health and the environment. Natural remediation of heavy metal-contaminated soils can be improved by immobilization techniques.

10.3.1 Immobilization Techniques

Ex situ and in situ immobilization techniques are practical approaches to remediation of metal-contaminated soils.

The ex situ technique is useful in areas where a large amount of contaminated soil must be removed from its place of origin, and its storage is connected with a high ecological risk. The main advantage of ex situ techniques is the fast and easy applicability. However, the disadvantages include (1) high invasivity to the environment, (2) generation of a significant amount of solid wastes (twice as large as volume after processing), (3) the by-product must be stored on a special landfill site, (4) in the case of changing of the physicochemical condition in the side product or its surroundings, there is serious danger of the release of additional contaminants to the environment, and (5) permanent control of the stored wastes is required. Soil remediation by conventional physicochemical technologies could be expensive; there is an interest in alternative remediation strategies.

In in situ technique, the fixing agent's amendments are applied on the unexcavated soil. The technique's advantages are (1) low invasivity, (2) simplicity and rapidity, (3) relatively inexpensive, (4) small amount of wastes are produced, (5) high public acceptability, and (6) envelop a wide spectrum of inorganic pollutants. The disadvantages of in situ immobilization are as follows (1) is only a temporary solution (contaminants are still in the environment), (2) the activation of pollutants may occur when soil physicochemical properties change, (3) the reclamation process is applied only to the surface layer of soil (30–50 cm), and (4) permanent monitoring is necessary (USEPA 1995; Martin and Ruby 2004). In situ immobilization technology often uses organic and inorganic amendment to accelerate the attenuation of metal mobility and toxicity (Mench et al. 2006). Specially, stabilization of contaminated soil by amendments or phytostabilization is a remediation technique that reduces the mobile fraction of heavy metals, which could contaminate groundwater or be taken up by soil organisms (Mench et al. 2000). In this respect, it is important remark that the study of solubility and bioavailability might be more important in remediation activities than the study of total or pseudo-total concentrations of these elements in contaminated soils, because they represent the most labile fractions subject to leaching and to being uptaken by plants and microorganisms (Adriano 2004).

10.3.1.1 Organic and Inorganic Amendments

There are different strategies for chemical immobilization of heavy metals in degraded soil. The use of soil amendments has been proposed as a low input alternative for remediation of metal-polluted soils. The primary role of immobilizing amendments is to alter the original soil metals to more geochemically stable phases via sorption, precipitation, and complexation processes (Hashimoto et al. 2009). The mostly applied amendments include clay, cement, zeolites, minerals, phosphates, organic composts, and microbes (Finžgar et al. 2006). In fact, in situ chemical immobilization decreases the concentration of dissolved contaminants by sorption or precipitation (Basta and McGowen 2004). It is well documented that some amendments (lime, phosphates, and organic and inorganic waste products) are effective in reducing mobility and availability of heavy metals in soils (Brown et al. 2005). In addition, their toxicity could be minimized by reducing their availability using organic and inorganic amendments (Adriano 2001; Basta et al. 2001). Generally, formation of insoluble metal element chemical species reduces both leaching through the soil profile and the labile pool available for biological interaction (Geebelen et al. 2003).

In particular, organic amendments, like mature compost, contain a high proportion of humified organic matter (humin and humic and fulvic acids). They could adsorb heavy metals temporarily through quelates' formation or by the formation of stable complexes sorbing them for a longer period (Basta et al. 2005). However, the abundant literature concerning the use of such amendments for metal immobilization is not conclusive, as contradictory results have been reported by different authors, depending upon various soil conditions, specific metals involved, origin, molecular size, and concentration of the organic matter, etc. For example, Yang et al. (2006) found that some organic ligands inhibited desorption of previously adsorbed Pb in soils at low ligand concentrations ($<10^{-3}$ mol l^{-1}), whereas a greater desorption was found at greater ligand concentrations. An extra important effect of organic amendment in soil is that it allows the recycling of nutrients and of organic matter present in them, and the improvement in soil physical properties (Sánchez-Monedero et al. 2004). On the other hand, inorganic amendments, such as water-soluble phosphates, provide long-term remediation through direct metal adsorption by the phosphate and precipitation of metals with solution phosphate (Adriano et al. 2004). In addition, inorganic amendments can also be used as a fertilizer to provide plant nutrients (Sharpley et al. 1999).

10.3.1.2 Phytoremediation

Due to their sessile nature, terrestrial plants have restricted mechanisms for stress avoidance, but during the course of evolution, some plant species have developed tolerance mechanisms to ensure the survival and breeding ability under elevated metal concentrations (Pastori and Foyer 2002) (Fig. 10.3). These adaptative responses of plants to heavy metal-contaminated environments are efficient

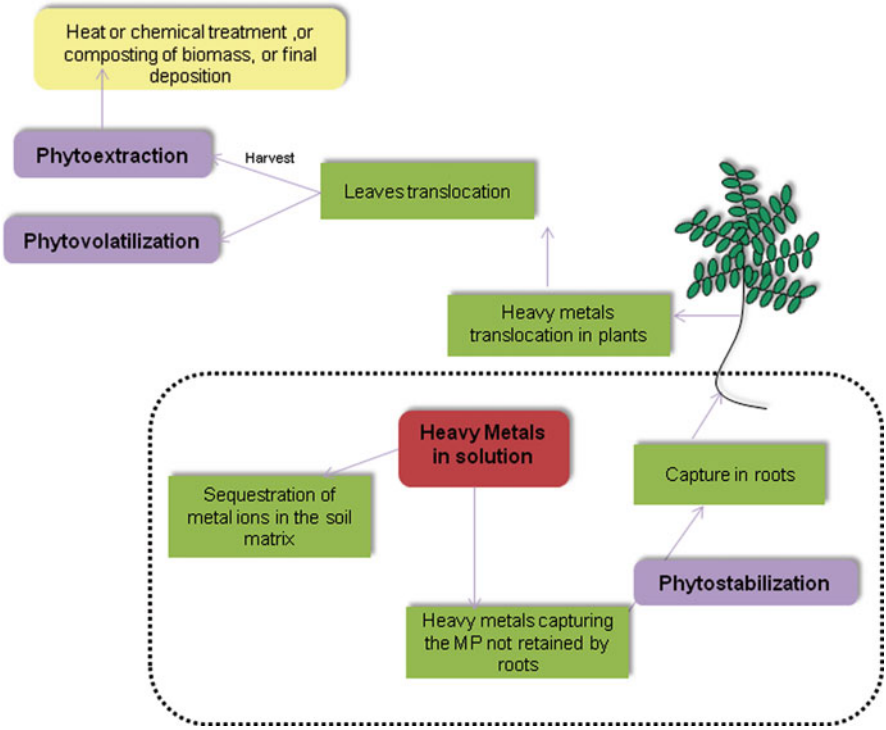

Fig. 10.3 General layout which explains the decontamination of heavy metal-contaminated soil in a natural process of phytoremediation. Adapted from Singh et al. (2003)

processes that include many physiological, molecular, genetic, and ecological traits (Mittler et al. 2004). The plant responses might differ as a function of doses, plant species, growing conditions, and phenology status (Sanitádi Toppi and Gabbrielli 1999).

Phytoremediation is an emerging technology, also called green remediation, that can be defined as an in situ remediation strategy that uses vegetation to remove, contain, or make environmental contaminants harmless (Helmisaari et al. 2007). There are four different plant-based technologies of phytoremediation, each having a different mechanism of action for remediating metal-polluted soil, sediment, or water: phytoextraction, phytovolatilization, phytostabilization, and phytofiltration (Sarma 2011). In particular, phytoremediation of heavy metal-contaminated soil aims to extract or inactivate metals in soils; so the most important technologies include phytoextraction (phytoaccumulation) and phytostabilization (Wei et al. 2008).

Phytoextraction: Phytoextraction is when plant roots uptake metal contaminants from the soil and translocate them to their above soil tissues. A plant used for phytoremediation needs to be heavy metal tolerant, grow rapidly with a high biomass yield per hectare, have high metal-accumulating ability in the foliar parts, have a profuse root system, and have a high bioaccumulation factor (Jadia and Fulekar 2008).

Phytostabilization: In Phytostabilization when certain plants immobilize soil contaminants (USEPA 2000), they are absorbed and accumulated by roots, adsorbed onto the roots, or precipitated in the rhizosphere. This reduces or even prevents the mobility of the contaminants preventing migration into the groundwater or air and reduces the bioavailability of the contaminant, thus preventing spread through the food chain. Plants for use in phytostabilization should be able to (1) decrease the amount of water percolating through the soil matrix, which may result in the formation of a hazardous leachate, (2) act as barrier to prevent direct contact with the contaminated soil, and (3) prevent soil erosion and the distribution of the toxic metal to other areas (Raskin and Ensley 2000). Phytostabilization can occur through the process of sorption, precipitation, complexation, or metal valence reduction. This technique is useful for the cleanup of Pb, As, Cd, Cr, Cu, and Zn (Jadia and Fulekar 2008). It can also be used to reestablish a plant community on sites that have been denuded due to the high levels of metal contamination. Once a community of tolerant species has been established, the potential for wind erosion is reduced, and leaching of the soil contaminants is reduced. Phytostabilization is advantageous because disposal of hazardous material/biomass is not required, and it is very effective when rapid immobilization is needed to preserve ground and surface waters (Jadia and Fulekar 2009; USEPA 2000). Therefore, sometimes it is extremely difficult to distinguish between direct and indirect responses if metal concentrations are too high or excessively prolonged. If there are metabolic alterations, these might reflect general failure of plant metabolism, but little is known about the earlier stages. Therefore, the characterization of heavy metal stress perception mechanisms should be undertaken in adequate experimental conditions, where we could learn about the primary cellular components involved. In fact, during the initial germination stage, there are many processes in which the presence of heavy metals will have a direct impact on seed viability and normal development of plants (Sobrero and Ronco 2004). Therefore, this stage is considered a critical phase in the life cycle of an individual (Veasey et al. 1999). Consequently, heavy metals' effects on initial germination stage might be assessed through chemical, biological, and toxicological data as well (Gruiz 2005). The use of phytotoxicity tests may offer a simple alternative to assess effects in early stage of plants.

The advantages of phytoremediation compared with classical remediation are as follows (1) it is more economically viable using the same tools and supplies as agriculture, (2) it is less disruptive to the environment and does not involve waiting for new plant communities to recolonize the site, (3) disposal sites are not needed, (4) it is more likely to be accepted by the public as it is more aesthetically pleasing then traditional methods, (5) it avoids excavation and transport of polluted media, thus reducing the risk of spreading the contamination, and (6) it has the potential to treat sites polluted with more than one type of pollutant. The disadvantages are as follows (1) it depends on environmental conditions (i.e., climate, geology, altitude, and temperature), (2) large-scale operations require access to agricultural equipment and knowledge, (3) success is dependent on the tolerance of the plant to the pollutant, (4) contaminants collected in senescing tissues may be released

back into the environment in autumn, (5) contaminants may be collected in woody tissues used as fuel, and (6) time taken to remediate sites far exceeds that of other technologies.

Plant species selection is a critical management decision for phytoremediation. Grasses are thought to be excellent candidates, because their fibrous rooting systems can stabilize soil and provide a large surface area for root–soil contact (Kulakow et al. 2000). The application of indigenous plant species for phytoremediation is often favored as it requires less management and acclimatizes successfully in native climate conditions and seasonal cycle. However, some exotic plant species may perform better in remediation of specific metals and can be safely used where the possibility of invasive behavior has been eliminated (USEPA 2000). Some important criteria in selecting plant species for phytoremediation are as follows:

- The levels of tolerance with respect to metal known to exist at the site
- The level of adequate accumulation, translocation, and uptake potential of metals
- High growth rate and biomass yield
- Tolerance to water logging and extreme drought conditions
- Availability, habitat preference (e.g., terrestrial, aquatic, semiaquatic)
- Tolerance to high pH and salinity
- Root characteristic and depth of the root zone

However, phytoremediation is energy efficient for remediating sites, and it can be used in combination with other remedial strategies as a finishing step to the remedial process.

10.3.2 Argentina's Phytostabilization Experiences

It is essential to use native plants for phytoremediation because these plants are often better in terms of survival, growth, and reproduction under environmental stress than plants introduced from other environments (Yoon et al. 2006). It is important to acknowledge the behavior that each species has into the region it belongs to (Brown et al. 2006). One way of contributing with the native phytogenetic resources conservation is to identify the tolerance to heavy metals of different species (Carpena and Bernal 2007). In order to achieve phytoremediation in the Argentinean pampas region, it is essential to assess the tolerance of species native to this area. In this respect, *Sesbania virgata* (Cav.) Poir., also known as Acacia Negra, a medium perennial shrub belonging to the legume family Fabaceae, is a native species from the Argentinean pampas region. Previous studies with *Sesbania* species in different areas have shown good results for phytoremediation of multicontaminated soils, accumulating significantly higher amounts of heavy metals in roots than in shoots (Ye et al. 2001; Chan et al. 2003; Tandy et al. 2006). Different species of *Sesbania* have been used for revegetation of

riparian forests, soil erosion control, and rehabilitation of degraded areas (Pott and Pott 1994). However, the level of accumulation of heavy metals differs between and within species (McGrath et al. 2002). That is why local remediation studies are important.

In a previous study, we found that *S. virgata* is capable of germinating and growing under heavy metal stress (Branzini and Zubillaga 2010). As a consequence, and because *S. virgata* is a pioneer species with rapid growth, we found interest in assessing the tolerance behavior of *S. virgata* (Vilela de Resende et al. 2000). In addition, in order to assess the tolerance behavior of *S. virgata*, the absorption, translocation, and growth performance of *S. virgata* in response to interactions between copper, zinc, and chromium in binary mixtures were evaluated (Branzini et al. 2012). In a pot experiment, heavy metals were added to soil (Typic Hapludoll) either individually or in binary mixture solutions of Cu, Zn, and Cr, in low or high doses (Low: Cu = 60, Zn = 125, and Cr = 50 (mg kg^{-1}); High: Cu = 700, Zn = 1,050, and Cr = 116 (mg kg^{-1})). This enrichment corresponds to the maximum levels of total HM that occur in the Pampas region (Llosa et al. 1990; Lavado et al. 1998; Giuffré et al. 2005). The *S. virgata* plants were allocated into 13 treatments as follows, and at harvest (30 days), plants were carefully removed and washed with deionized water to remove any attached particles. The heavy metal transfer from a contaminated soil to plants and into plant tissues is discussed in terms of the Bioconcentration Factor (BCF) and the Transfer Factor (TF) [see (10.1) and (10.2)]:

$$\mathbf{BCF} = [\mathbf{HM}]_{\mathbf{plant}} / [\mathbf{HM}]_{\mathbf{dry\ soil}}, \tag{10.1}$$

$$\mathbf{TF} = [\mathbf{HM}]_{\mathbf{shoots}} / [\mathbf{HM}]_{\mathbf{roots}}, \tag{10.2}$$

where $[HM]_{plant}$ is the concentration of heavy metals in plant tissues, $[HM]_{dry\ soil}$ is the initial concentration in the environment, $[HM]_{shoots}$ is the heavy metal concentration in the aboveground part of the plant, and $[HM]_{roots}$ is the concentration in the roots.

The results showed that plant shoots accumulated lower concentrations of heavy metals than plant roots (Fig. 10.4). The highest concentration of Cu in both shoot/leaves and roots was observed when Zn was added simultaneously at high doses (Fig. 10.4a). Consequently, these results suggest that the simultaneous presence of Cu and Zn increases the extraction capacity of *S. virgata* plants, indicating synergistic effects between them. This finding is in agreement with that found by Luo and Rimmer (1995), who demonstrated that the increase in Zn uptake due to the addition of Cu is approximately 20 % and that Cu uptake also increases with Zn addition.

In addition, as the levels of soil heavy metals increased, either alone or in binary mixtures, their concentration in plant tissues increased significantly. Concentration of Cu in aboveground and root biomass was found to be significantly high when it was alone or in mixture, at the higher doses (Table 10.1). Total Zn concentration

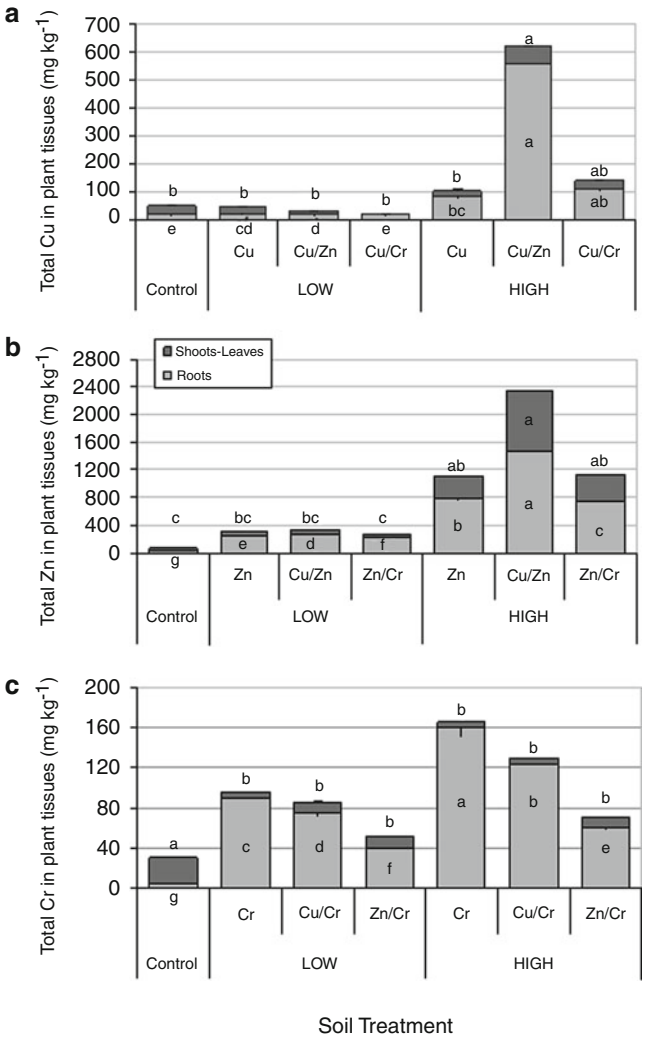

Fig. 10.4 Total concentration and distribution of heavy metals in shoots and roots of *Sesbania virgata* plants. *Vertical bars* represent standard deviations. The means followed by the same latter (a–e) were not significantly different at $p < 0.05$

had a pattern of variation different from that of Cu. At high doses, the concentration in shoots/leaves was higher than at low doses only with Cu (Fig. 10.4b; Table 10.1). In contrast, Cr concentration was higher in roots of *S. virgata* in the individual treatment at high doses. A possible explanation for this trend is that the sorption capacity of each metallic cation of the mixture might decrease in competitive processes (Flogeac et al. 2007) (Fig. 10.4c; Table 10.1).

The transfer factor (TF) determined for *S. virgata* tissues showed a similar behavior for Cu, Zn, and Cr. Generally, in all treatments, TF values obtained for

Table 10.1 Comparison between contamination levels in shoot/leaves and roots of *Sesbania virgata* plants, when HM were added individually and in mixture

ANOVA (*p values*)	Cu	Zn	Cr
Shoots and leaves	0.0042	0.0006	0.004
Roots	<0.001	<0.001	<0.001
Contrasts			
Shoots and leaves			
Cu and Cu/Zn low vs. Cu and Cu/Zn high	<0.001		
Cu and Cu/Cr low vs. Cu and Cu/Cr high	<0.001		
Cr and Zn/Cr low vs. Cr and Zn/Cr high			0.0157
Roots			
Cu and Cu/Zn low vs. Cu and Cu/Zn high	0.0025		
Cu and Cu/Cr low vs. Cu and Cu/Cr high	0.0234		
Zn and Cu/Zn low vs. Zn and Cu/Zn high		0.0002	

Table 10.2 TF and BCF in different tissues of *Sesbania virgata* under soil HM treatments

		Cu			Zn			Cr		
HM levels		TF	Root BCF	Shoot BCF	TF	Root BCF	Shoot BCF	TF	Root BCF	Shoot BCF
Control		2.18	1.41	3.07	1.11	0.78	0.87	6.48	0.68	4.40
Cu	Low	1.11	0.34	0.41						
	High	0.23	0.12	0.03						
Zn	Low				0.32	2.03	0.64			
	High				0.42	0.75	0.32			
Cr	Low							0.05	1.81	0.09
	High							0.03	1.39	0.05
Cu/Zn	Low	0.55	0.34	0.19	0.26	2.18	0.56			
	High	0.11	0.80	0.09	0.59	1.40	0.83			
Cu/Cr	Low	0.18	0.29	0.05				0.11	1.52	0.17
	High	0.30	0.16	0.05				0.05	1.08	0.06
Zn/Cr	Low				0.30	1.79	0.55	0.28	0.8	0.22
	High				0.51	0.72	0.37	0.14	0.53	0.08

Note: TF Translocation factor, *BCF* Bioconcentration factor

the above- and underground part of the plants were <1.0 (Table 10.2). Therefore, all heavy metals were accumulated in greater concentrations in plant roots. On the other hand, the BCF average in roots was Zn (1.4) > Cr (1.1) > Cu (0.5), the roots being more effective than shoots in accumulating heavy metals (Table 10.2). Similarly, to that found by other authors, this seems to indicate that *S. virgata* would have a higher bioaccumulation potential of Zn and that it is more effective in removing it from soils (Zhang et al. 2010). Absorption and accumulation of heavy metals in plant tissues depend upon many factors (Sarma 2011). A significant accumulation of Cu, Zn, and Cr in the root system compared with the shoot parts of *S. virgata* may indicate a higher efficiency in restraining the translocation and/or low capacity for controlling their absorption, preventing them from reaching metabolically more

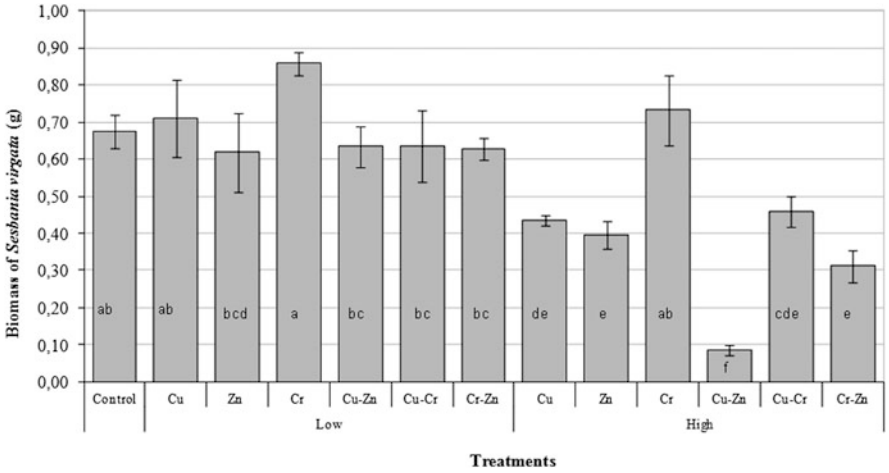

Fig. 10.5 Effects of heavy metals stress on biomass of *Sesbania virgata*. Data are means ± 1 S.D. Means followed by the same letter (a–e) were not significantly different at $p < 0.05$

active tissues of the shoot parts (Zhang et al. 2010). These results are in agreement with those of Sinha and Gupta (2005), who demonstrated that the accumulation of heavy metals varies from one part to another within the plant tissues.

In general, plants grown in treatments with low levels of heavy metals had the highest biomass performance ($p < 0.001$, Fig. 10.5) compared to treatments with high levels. In particular, the maximum decrease in *S. virgata* biomass was noticed in the mixture of Cu and Zn at high levels (87.4 %), having, also, a delay in their development. These results suggest that the type of interactions between the constituent metals in the mixture is a synergistic or additive response. In conclusion, the results suggest that the uptake of one metal is affected by the presence of other metals. In addition, *S. virgata* plants have the capacity to tolerate and stabilize high concentrations of Cu, Zn, and Cr in soils. It is assumed that roots may play an important role in metal retention by preventing an excessive and toxic accumulation in shoots. The co-presence of metals resulted in a greater reduction in *S. virgata* biomass than the presence of a single metal. In particular, when Cu and Zn were present at high levels this reduction was higher, thus suggesting a synergistic or additive response. Based on this experiment, and in view of their uptake capacity and tolerance, we propose that *S. virgata* plants can be used for phytostabilization of metals in contaminated soils.

10.4 Recycling Heavy Metals

According to the Environmental Protection Agency, hazardous wasted reuse, recycling, and reclamation (recycling metals) can avoid environmental hazards, protect natural resources, and reduce the nation's reliance on raw materials and energy.

Consequently, in order to reduce significantly the loss of heavy metal to the environment by human activities, recycling is an interesting part solution. In addition, this action could avoid the new metal entering into circulation. As a result, a metal-containing waste could be converted to product. However, reality is that significant quantities of heavy metals will never be collected for recycling by the present waste management systems. Thus recycling will not prevent a continued release to the environment of heavy metals in circulation in the technosphere.

10.5 Conclusions

The effects of mixtures of heavy metals' incorporation into soil and their toxic properties are important challenges that we have to outface. In addition, the utilization of in situ technologies to remediate the effects has to be carefully selected due to the objective of remediation. Amendments and phytostabilization could adsorb, bind, or co-precipitate the contaminating metals and/or can use plants for immobilization of toxic metals. Consequently, stabilization of heavy metals in soil appears as a low-cost alternative and supposes an attractive and emerging technology for site restoration. However, it has to be clear that these strategies require monitoring process. The use of plants in combination with chemical amendments in designing low-cost treatment system is still a challenge in environmental managements. In fact, in Argentina, there are some species like *Sesbania virgata*, which have high capacity to tolerate and accumulate heavy metals in their tissues, being useful for phytostabilization of contaminated soils with mixtures of heavy metals.

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Chapter 11
Flax (*Linum usitatissimum* L.) and Hemp (*Cannabis sativa* L.) as Fibre Crops for Phytoextraction of Heavy Metals: Biological, Agro-technological and Economical Point of View

Miroslav Griga and Marie Bjelková

11.1 Introduction

The technology of heavy metals (HMs) phytoextraction from the polluted soils should include several components in order to be exploitable and cost efficient. First one is a biological component, which represents available plant species exhibiting high/good HMs accumulation and tolerance. Such candidate species should produce high above-ground biomass and the metal element of interest should be easily transported from roots to above-ground harvestable organs. From the genetic point of view, the culture crops have advantage (as compared to wild species) that they represent genetically homogenous and stable populations (clone, line and variety) with optimised growth and development parameters provided by breeding process. Second one is a technological component, which involves complex technology of growing, integrated plant protection and harvest (using efficient field machinery), and regulation of HMs bioavailability and uptake by proper agrotechnological treatments. The very important point is the possibility of further industrial processing of HMs-contaminated biomass. Finally, third one is an economical component which includes cost of phytoextraction, time of phytoextraction (cleaning of particular site to desired/acceptable level of metal element), cost of handling with HMs-contaminated biomass, and reduction of overall costs of phytoremediation process by production of added value products from HMs-contaminated biomass. If one component of the scheme suffers from some shortcomings/limits, it should be compensated by advantages of other components.

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Table 11.1 Yield of above-ground biomass and fibre of important fibre crops

Plant species	Common name of plant/product	Biomass yield (tonne ha^{-1})	Fibre yield (tonne ha^{-1})
Linum usitatissimum	Flax	6.5–7.5	1.1–1.5
	Linseed	2–5	0.2–0.5
Cannabis sativa	Hemp	8–15	1.5–1.9
Hibiscus cannabinus	Kenaf	5–12	0.4–1.3
Gossypium hirsutum	Cotton	1–3.4	0.3–1.1
Corchorus olitorius	Jute	30–35	1.4–4
Boehmeria nivea	White ramie	1.7–2	0.3–0.5
Asclepias syriaca	Common milkweed	12.3	1.4
Agave sisalana	Sisal	5,000 Plants	2.8–5
Musa textilis	Banana/abaca	–	5
Cocos nucifera	Coconut/coir	1,000 nuts	0.1
Crotalaria juncea	Sunhemp	7–10	0.6–0.8

Fibre crops may represent a good compromise between the lower HMs accumulation potential and the possibility of industrial (non-food) processing of contaminated biomass. Fibre crops are world-wide distributed group of plant species belonging taxonomically to various plant families. The common denominator is their use of above-ground biomass (Table 11.1) for mainly industrial (non-food) or energy purposes. They include approximately 2,000 species—annual and perennial—belonging to monocotyledonous as well as dicotyledonous plants. About 20 species have got an economical (some of them local) importance. Majority of fibre species is grown in tropical and subtropical zones. Cellulose, a natural polymer with high strength and stiffness per weight, is the building material of long fibrous cells, which can be found in the stems, the leaves or the fruits/seeds of fibre plants. Thus, based on the fibrous cells localisation within the plant we can recognise bast fibre species (e.g. flax, hemp, jute, kenaf, ramie and sida), leaf fibre species (sisal, banana and palm) and fruit/seed fibre species (cotton, coconut, kapok and luffa) (Brouwer 2000). World acreage and production of the most important fibre crop commodities are illustrated in Table 11.2. During last 20 years, the fibre crops have been also considered as potential candidates for phytoremediation, particularly for phytoextraction of heavy metals from contaminated soils (Griga et al. 2003a, b; Linger et al. 2002). Within fibre crops of temperate and subtropic zone, the flax/linseed and hemp represent economically the most important species and also the majority of heavy metal-related data were obtained and published in these two fibre crops. As the main portion of HM-data in flax and hemp have been published in national scientific literature, this review tries to summarise all this available literature in order to bring a complex view of recent knowledge of flax and hemp phytoremediation potential and also to critically assess original expectations versus real situation of recent days.

Table 11.2 World production and acreage of important fibre crops

Fibre crop/product	Production (t)	Area (ha)
Agave fibres nes	34,210.00	43,440.00
Fibre crops nes	312,840.00	361,608.00
Flax fibre and tow	622,200.00	231,841.00
Hemp tow waste	80,048.00	53,652.00
Jute	3,055,856.90	1,303,354.30
Kapok fruit	358,500.00	170,300.00
Manila fibre (Abaca)	95,222.00	155,900.00
Other bastfibres	247,930.00	180,295.00
Ramie	118,000.00	75,340.00
Seed cotton	68,303,311.36	32,009,033.39
Sisal	361,307.06	417,510.00

Source: FAOSTAT (2010)

11.2 Flax/Linseed (*Linum usitatissimum* L.): Botanical Characterisation and Economical Uses

Flax (*Linum usitatissimum* L.) is one of the oldest domesticated crops grown for seed, oil and fibre use. Flax was cultivated in Egypt and Samaria 10,000 years ago (Zohary and Hopf 2000) and is the first fibre crop of the ancient Near East (Abbo et al. 2010). First cultivation of flax in Europe was in Neolithic era in the area of recent south Germany, Switzerland, Ireland, Scotland, Sweden, Denmark and Poland. The ancient Egypt was the most important place of flax cultivation with the written evidence about flax retting and dying. About 1200 BC, the Jewish during exodus from Egypt brought the knowledge on flax cultivation and processing to new settlements (Palestina, France, England and Ireland; Mojžíš 1988). Vavilov (1926) proposed the centre of origin of flax is in the Middle East, although secondary centres of diversity are identified in the Mediterranean Sea, Ethiopia, Central Asia and India.

The origin of flax (*L. usitatissimum* L.) is uncertain. The genus *Linum*, belonging to the family of *Linaceae* comprising 22 genera with over than 250 species, is mainly spread in the Mediterranean in temperate climate prairies of north hemisphere. Cultivated flax is related to *L. bienne* Mill. (syn. *L. angustifolium* Huds). The subsection *Linum* contains the cultivated species *L. usitatissimum* L. and the ornamentals *L. grandiflorum* Desf. and *L. perenne* L., but only *L. usitatissimum* has economic importance. The species is self-pollinated and has the number of chromosomes $2n = 30$ (Muravenko et al. 2003; Gill 1987). While *L. usitatissimum* is an annual crop species, the wild species can also be biannual or perennial. Flax *L. bienne* is probable progenitor of cultivated flax *L. usitatissimum* based on previous characterisations (cytology, morphology and molecular analysis) (Diederichsen and Hammer 1995; Muir and Wescott 2001; Fu 2005; Allaby et al. 2005; McDill JR 2009; Uysal et al. 2010).

L. usitatissimum L. is represented by two technological types—flax for fibre production and linseed for seed production. The flax stem should be long, straight,

thin and slender and with short top branching (Štaud et al. 1997). Leaves are simple, sessile, linear-lanceolate with entire margins. The inflorescence is a loose terminal raceme or cyme. Flowers are borne on long erect pedicels, are hermaphrodite, hypogynous and are composed of five sepals, five petals (blue), five stamens and a pistil compound of five carpels each separated by a false septum. The fruit is a capsule, composed of 5 carpels and may contain up to 10 seeds. The seed is oval, lenticular, 4–6 mm long with a smooth, shiny surface, brown to light-brown in colour. Root system of flax is shallow and it consists of a main root and many lateral roots. The root is thin with fibrous branches with length 0.9–1.2 m. Main root is straight and secondary roots are further branching (Gill 1987; Mojžíš 1988). The cross-section of flax stem is usually round to oval and stem width is 1.2–2 mm. The height of flax plants is 1,200–1,500 mm. The linseed stem should be thicker, shorter, with more robust top branching. Seeds contain 20–25 % protein and 35–45 % oil rich in unsaturated fatty acids (FA). Standard FA composition in commercial flax and linseed cultivars is about 6 % palmitic (16:0), 2 % stearic (18:0), 16–20 % oleic (18:1), 13–18 % linoleic (18:2) and 52–60 % α-linolenic (18:3) acids (Gill 1987; Dir 94-10; Pavelek et al. 2011).

For both technological types—flax and linseed—there are partly different ways of cultivation and use of main product and by-product. After scutching process, the long fibre and tow is mainly used for the manufacture of tablecloths, bed linen, decorative fabrics and as an additive to cotton or synthetic fibres. It has extensive application in the technical use in the manufacture of solid yarns, twine, cordage, hoses and tires. Wood (shives) is used in building and furniture industry. Flax seed is used as food, pharmaceutical, as a component in compound feed and its oil for the production of varnishes and paints. Linseed provides more options for use than most other crops (Singh et al. 2011). The main product of linseed is seed. Linseed is used whole or slightly crushed for the production of bread and rolls, adding to the dough, making bakery products (Gambus et al. 2004). Furthermore, the seed is used in the feed industry and the production of oil, which is used in the oleochemical industry for the manufacture of soaps, paints, biodiesel (Lingaraju et al. 2012), varnishes, slow drying lacquers, and also in medicine, where it is known for its positive effect on the degradation of fatty acids and cholesterol (Kolodziejczyk and Kozlowska 1993; Gambus et al. 2004). The components of feed mixtures are pressed parts and cake, which contain mucilage dietetic substances and proteins. Using linseed protects the stomach against agents that damage the lining of the endocrine ulcers due to the content of mucilage. Linseed stem and fibre are products (Štaud et al. 1997) for use as bedding for cattle fattening, and in many industries, such as construction, paper, composite materials (Cappelletto et al. 1998; Bledzki and Jaszkiewicz 2010; Kwiatkowska et al. 2012), packing cloth, twine, filter covers, geotextiles and replacement of plastic materials. Shive is also used in the manufacture of composite plates or pressing seed pots and packaging materials (Wedler and Kohler 1993/1994). Stem may be utilised in combustion and heat energy production in local facilities (loosely pressed larger packages) or modified into briquettes for small consumers (Štaud and Bjelková 1997). Thus, flax/linseed as a multipurpose

crop provides 100 % utilisable raw material with fully biodegradable waste and no harmful residues.

11.3 Hemp (*Cannabis sativa* L.): Botanical Characterisation and Economical Uses

The exact origin of *Cannabis* is unclear, because it was dispersed across Eurasia by humans very early in pre-history. However, Central Asia offers by far the most plausible location for the origin of *Cannabis*. From Central Asia *Cannabis* was carried out throughout East Asia, South Asia and Europe which served as primary centres of domestication and secondary gene pools (Clarke 1999). Recently, *Cannabis* is dispersed and grown throughout the world from subarctic to tropical regions. China has produced the oldest archaeological and historical evidence (around 2700 BC) of *Cannabis* cultivation and use (Ranalli and Venturi 2004). It is likely that the Chinese were the first to use wild *Cannabis* and domesticate it for its fibre and seed, and that Indians were the first to use it and domesticate it for its psychoactive properties (Abel 1980; Clarke 1999).

Cannabis and *Humulus* are the only genera in the family *Cannabaceae*. There are several taxonomic views on the genus *Cannabis*—the most common ones consider genus *Cannabis* as monospecific, i.e. *C. sativa* with two subspecies (Small and Cronquist 1976) or split *Cannabis* into three species *C. sativa*, *C. indica* and *C. ruderalis* (Schultes et al. 1974), with the last-mentioned species to be a truly wild taxon and probable ancestor to cultivated hemp varieties (Clarke 1999). Recent application of biochemical (Hillig 2005) and molecular markers (Faeti et al. 1996; Kojoma et al. 2002; Mandolino and Ranalli 2002; Gilmore and Peakall 2003) for *Cannabis* germplasm evaluation promises to bring new light into *Cannabis* phylogeny and taxonomy.

Cannabis is a medium to tall, erect, annual herb. However, environmental influences on the growth habit of *Cannabis* are very strong. Provided with an open sunny environment, light well-drained soil, and ample nutrients and water, *Cannabis* can grow to a height of 5 m in a 4- to 6-month growing season (Clarke 1999), thus forming a huge above-ground biomass (Table 11.1). In contrast, when growing in arid locations with limited soil nutrients, *Cannabis* plants develop minimal foliage and may mature and bear seed when only 20 cm tall. Plant density in a square unit affects significantly plant architecture—when planted in close stands (for fibre production; ca. 100 plants per m^2), hemp plants do not branch but grow as tall, thin, straight stalks. When grown for seed production (ca. 4 plants per m^2), hemp plants are extensively branched. *Cannabis* is normally a dioecious plant (male and female flowers develop on separate plants), although monoecious individuals with flowers of both sexes on one plant are occasionally found. Before flowering, the sexes of *Cannabis* are indistinguishable, except for general trends in growth habit in certain strains as height and extent of branching. *Cannabis* is

anemophilous (wind pollinated). *Cannabis* exhibits a dual response to day length. During the first 2 or 3 months of growth it responds to increasing daylength with more vigorous vegetative growth, but later it requires shorter days (or more accurately long nights) to flower and complete its life cycle (Clarke 1999).

Similarly as flax, hemp represents a multipurpose crop. The strong fibres, edible fruits/seeds and psychoactive drugs produced by *Cannabis* have attracted humans since Neolithic times (Clarke 1999; Ranalli and Venturi 2004). Hemp fibre is traditionally used as a raw material for paper and textile production. The last decades showed a renewed interest in natural fibres and their novel use in automotive industry, furniture and building industry, mainly in the form of composite materials (Brouwer 2000; Karus and Vogt 2004). The oil content of hemp seed is high (35 %) and comparable in yields per hectare with rape and sunflower oil; in addition, it has important pharmaceutical properties. High above-ground biomass of hemp may be also used advantageously as a combustible raw material for energy production (Scholz and Ellebrock 2002; Ranalli and Venturi 2004). Thus, due to the agricultural benefits (weed control, pest and disease resistance, pesticide elimination, soil improvement by means of crop rotation, high biomass production with low inputs), the plethora of industrial uses (Karus and Vogt 2004) and a limited environmental impact, hemp is potentially profitable crop, having the right profile to fit into sustainable (both conventional and organic) farming systems, promoting long-term land management strategy (Ranalli 1999; Ranalli and Venturi 2004).

11.4 Flax, Hemp and Heavy Metals Studies: Hygienic Versus Bioremediation Aspect

Prevalence of industrial use and relatively high uptake of heavy metals from the soil (flax often accumulates in its tissues higher concentrations of e.g. Cd as compared to soil Cd content—Gaudchau and Marquard 1990; Schubert 1992; Böhm et al. 1992; Böhm and Marquard 1993a, b; Moraghan 1993; Schneider and Marquard 1996) predeterminates this crop to be a potential candidate for phytoremediation of soils polluted by heavy metals. The beginning of systematic research of flax in relation to heavy metals was dated 25 years ago (Klein and Weigert 1987) and it may be characterised particularly by two points of view (1) hygienic aspect connected with the effort to minimise heavy metal accumulation in seeds and (2) Cd was the most frequently studied element (less information available on other heavy metals). Nevertheless, first reports on possibilities of utilisation of flax (and fibre crops generally) for phytoremediation appeared very soon after these "hygienically oriented studies" (Böhm et al. 1992; Kozlowski et al. 1993, 1993/1994; Mankowski et al. 1994; Baraniecki et al. 1995). In fact, the monograph on linseed (Gill 1987) as well as the comprehensive review on mineral nutrition of flax and linseed (Hocking et al. 1988) did not contain any data about heavy metal effects on flax/linseed growth or on their content in plant organs.

The leading idea and common objective of early reports on linseed and heavy metals was to eliminate or minimise the heavy metal (mainly Cd) accumulation in the seeds and subsequent food-chain transfer to human body. Because Cd has a half-life of ca. 20 years in the human body, the consumption of Cd-contaminated foods may lead to chronic toxicity. According to WHO (1972), the maximum tolerable intake limit for an adult is 60–70 μg Cd per day. Thus, the accepted dietary critical value is 0.3 mg Cd kg^{-1} DW of the flax seed according to German Richtwer (Klein and Weigert 1987; Anonymous 1988; Marquard et al. 1990), maximum permitted concentration of Cd proposed for confectionery linseed traded on the international market is even lower—0.25 mg Cd kg^{-1} DW (Codex Alimentarius Commission 1993; Hocking and McLaughlin 2000). Lukipudis (2001) studied the contamination of flax fibre by Cu, Cd and Pb in polluted areas in Bulgaria and found the exceeding of HAC (highly admissible concentration) of complex contamination of fibre by heavy metals according to DIN/ISO standards as related to clothing production for adults and children. The solution of the problem may be reached by selection of genotypes with naturally low uptake and translocation of heavy metals, by avoiding the growing areas heavily polluted by heavy metals or by managing agricultural systems preventing the heavy metal ions bioavailability to flax plants (Grant et al. 1998).

Wide possibilities of utilisation of flax (and other fibre crops) for industrial (non-food) products resulted in the half of nineties of the last century in speculations and first experiments directed on phytoremediation potential of flax for heavy metals (Böhm et al. 1992; Kozlowski et al. 1993, 1993/1994; Mankowski et al. 1994; Baraniecki et al. 1995). Thus, the demands for uptake and accumulation of heavy metals by flax plants changed into quite contrast to hygienic aspects. It was known from previous studies that flax accumulates in seeds relatively high heavy metal concentrations as compared to other grain crops and even evidently elevated Cd content as compared to soil Cd concentration (Gaudchau and Marquard 1990; Schubert 1992; Böhm et al. 1992; Böhm and Marquard 1993a, b; Moraghan 1993; Schneider and Marquard 1996). The aim of studies was to prove if flax may uptake and accumulate in aboveground parts elevated amounts of heavy metals and—at the same time—not to decrease the yield and quality of harvested raw material for subsequent industrial processing. It was necessary to find if there is a genotype variation in uptake, translocation and accumulation of heavy metals and by which agrotechnological treatments is possible to increase the bioavailability and uptake of heavy metals by flax roots. Research activities in that way were substantial in Poland, where besides of pot experiments (Kozlowski et al. 1993a, b; Mankowski et al. 1994), the first on site studies started—e.g. experiments on polluted soils near Copper Smelting Factory in Glogów (Baraniecki et al. 1995, 2001; Grzebisz et al. 1997a, b; Grabowska and Baraniecki 1997). An overview of Cd (as the most frequently studied toxic metal element) research in flax is provided in Table 11.3.

There was an absence of knowledge on hemp as related to heavy metals up to the beginning of the 1990s of the last century (similar situation as in flax). The hemp research was mainly concentrated on drugs and oil content/composition (seed

Table 11.3 The summary of Cd uptake and accumulation by flax/linseed (*Linum usitatissimum* L.)

Aim of study	Cd concentration in substrate (mg kg^{-1})	Cd concentration in plant tissue (mg kg^{-1} DW)				Reference
		Seed	Capsule	Stem	Root	
Cd in seeds of linseed; various field locations in Germany; genotype variation in Cd uptake; *hygienic aspect*	0.22–0.25	0.1–1.7	n.d.	n.d.	n.d.	Marquard et al. (1990)
Cd in linseed organs; pot experiment, greenhouse, soil; *hygienic aspect*	<0.10–0.20	0.4–0.6	n.d.	0.4–0.5	n.d.	Schubert (1992)
Cd in seeds of linseed; pot experiment, greenhouse, soil, effect of Zn, N, P, Fe; *hygienic aspect*	0.15–1.00	0.16–6.26	n.d.	n.d.	n.d.	Moraghan (1993)
Cd uptake and accumulation in linseed organs; soil, growth chamber; *hygienic aspect*	0.12–0.34	0.12–1.1	n.d.	0.27–1.30	0.42–1.12	Cieslinski et al. (1996)
Cd in linseed organs; pot experiment, greenhouse, soil; genotype variation in Cd uptake; *hygienic aspect*	0.13–0.35	0.20–0.60	0.40–1.00		0.20–0.60	Schneider et al. (1996)
Cd in linseed organs; five field locations in Germany; genotype variation; *hygienic aspect*	0.12–1.30	0.05–1.30	0.30–0.50		0.33–0.53	Schneider and Marquard (1996)
Cd, Cu, Pb, Zn, Mn, Ni, Cr in fibre flax organs; effect of industrial pollution on heavy metal accumulation; *ecological* (*phytoremediation*) *aspect*	Soils contaminated by copper factory	0.18–0.33	0.15–0.39	0.35–0.82	n.d.	Straczynski and Andruszczak (1996)
Effect of genotype, explant and Cd stress on callus growth and regeneration in vitro as a background for Cd-tolerance selection in linseed; *ecological* (*phytoremediation*) *aspect*	50–2,000 μM	n.d.	n.d.	n.d.	n.d.	Chakravarty and Srivastava (1997a)
In vitro regeneration of Cd-tolerant linseed plants; Cd × Zn interaction; stress-induced marker: peroxidase; *ecological* (*phytoremediation*) *aspect*	0.1–0.2 μM	n.d.	n.d.	0.75–1.8	3.5–5.2	Chakravarty and Srivastava (1997b)
Screening of low Cd linseed genotypes; field experiment, USA; genotype variation in Cd uptake; *hygienic aspect*	0.12–0.15	0.14–1.55	n.d.	n.d.	n.d.	Li et al. (1997)

Cd in seeds of linseed; pot experiment, greenhouse, soil; genotype variation in Cd uptake; *hygienic aspect*	0.28–1.00	0.21–0.33	0.21–0.26	0.6–0.7	n.d.	Becher et al. (1997)
Cd and Zn in seeds of linseed; three field locations in Canada; effect of fertiliser with P and Zn; *hygienic aspect*	0.24–0.37 (mg ha^{-1})	0.28–0.53	n.d.	n.d.	n.d.	Grant and Bailey (1997)
Cd and Zn in seeds of linseed; three field locations in Canada; effect of N and P fertilisation; genotype variation; *hygienic aspect*	0.10–0.27	0.25–1.76	n.d.	0.32–2.30	n.d.	Grant et al. (2000)
Cd in seeds of linseed; pot experiment, greenhouse, soil; genotype variation in Cd uptake; *hygienic aspect*	0.17–0.22	0.23–0.55	0.34–0.63	n.d.	n.d.	Hocking and McLaughlin (2000)
Cd, Pb, Cu, Zn, Fe, Co, Cr, Hg in flax fibres and products; effect of growth regulators on heavy metal content	Data not available	n.d.	n.d.	0.07–0.15 fibre; flax products	n.d.	Belopuhov et al. (2001)
Cd, Pb and Cu in fibre flax seeds and fibres; field experiment—soil contaminated by complex of heavy metals; genotype variation in heavy metal uptake; *hygienic aspect*	Data not available	1.53–1.73	n.d.	2.70–3.50 fibre	n.d.	Lukipudis (2001)
Uptake and accumulation of Cd and Pb in flax and linseed organs; tissue culture, greenhouse and field-simulated experiments; genotype variation; *phytoremediation aspect*	0.35–200.00 (soil) 5–100 (agar medium)	0.13–5.74	0.10–26.35	0.30–23.79	0.29–22.28	Bjelkova et al. (2001)
Determination of the quantities and the sites of accumulation of Pb, Cu, Zn and Cd in the vegetative and reproductive organs of fibre crops (flax, hemp and cotton); *ecological (phytoremediation) aspect*	2.5–12.2 (soil)	0.52–2.30	n.d.	1.63–7.27	1.94–8.69	Angelova et al. (2004)
Monitoring of phytochelatins induction in Cd-treated cell suspension cultures of flax	10–100 µM (liquid medium)					Vrbová et al. (2009)

(continued)

Table 11.3 (continued)

Aim of study	Cd concentration in substrate (mg kg^{-1})	Cd concentration in plant tissue (mg kg^{-1} DW)				Reference
		Seed	Capsule	Stem	Root	
Large scale screening of heavy metal tolerance in flax/linseed *in vitro*; *ecological* (*phytoremediation*) *aspect*	0–50 (mg l^{-1}) (agar medium)	n.d.	n.d.	n.d.	n.d.	Smýkalová et al. (2010)
Screening of the effect of preceding crop and P-fertilisation on Cd and Zn concentration in flaxseed; agronomical pre-treatments	72–134.8 (soil)	0.2–0.372	n.d.	n.d.	n.d.	Grant et al. (2010)
Screening of heavy metals toxic effect on seed germination in 23 cultivars of Flax (*Linum usitatissimum* L.); *ecological* (*phytoremediation*) *aspect*	0.01–1 mM	n.d.	n.d.	n.d.	n.d.	Soudek et al. (2010)
Accumulation of Cd by flax and linseed cultivars in field-simulated conditions; *ecological* (*phytoremediation*) *aspect*	10–1,000	0.18–3.54	0.16–3.91	0.39–10.41	1.45–60.22	Bjelková et al. (2011a)
Effect of sewage sludge in soil on Cd accumulation in the *Linnum usitatissimum* L.; *ecological* (*phytoremediation*) *aspect*	n.d. (mixture of sewage sludge/soil)	0.07–0.16	0.26–0.54	0.2–0.57	0.29–0.38	Bjelková et al. (2011b)
Studying the mechanisms of intracellular detoxification of Cd in flax seedlings; *ecological* (*phytoremediation*) *aspect*	50–1,000 µM (agar medium)	n.d.	n.d.	n.d.	0.4–1.9 (mg g^{-1})	Najmanova et al. (2012)
Enhancing of the Cd accumulation in *Linum usitatissimum* L. by overproduction of metallothioneins via transformation process; *ecological* (*phytoremediation*) *aspect*	20–360 (soil) 15 (agar medium)	n.d.	n.d.	3–42 (mg g^{-1})	n.d.	Vrbová et al. (2012)

strains) or fibre content/composition (fibre strains), but not on mineral composition of hemp plants (viz. monograph edited by Ranalli 1999). First reports dealing with heavy metals uptake/accumulation by hemp were concentrated on the agrotechnological treatments (e.g. fertilisation, liming) affecting heavy metal phytoavailability (Jurkowska et al. 1990, 1992; Jasiewicz 1991) and toxic effect of metal elements on hemp plants (Gorlach and Gambuś 1992; Gorlach 1994). These studies were immediately followed by the idea for the potential use of this crop to clean industrially polluted soil with possible use of contaminated biomass for industrial products (Mankowski et al. 1994; Baraniecki et al. 1995). Recently, several reports have seriously studied hemp phytoextraction potential of heavy metals (Löser et al. 2002; Linger et al. 2002; Angelova et al. 2004; Antonkiewicz et al. 2004; Kos and Leštan 2004). To date, ca. 30 journal papers (mostly national journals), book chapters or conference abstracts have been published dealing with hemp and heavy metals. Main portion of data includes Cd, Pb, Cu, Zn, Ni and Cr and they are summarised in Table 11.4.

11.5 Heavy Metal Phytoextraction by Flax and Hemp

11.5.1 Genetic Variation in Uptake, Translocation and Accumulation of Heavy Metals

Both flax and hemp do not represent HMs-hyperaccumulators—this fact may be compensated by production of high above-ground biomass (particularly in hemp; viz. Table 11.1). Nevertheless, the choice of genotypes/lines/varieties with higher accumulation of particular metal elements (Cd, Pb, Zn, Ni) would be beneficial for the final impact of phytoextraction technology. Unfortunately, no breeding work in flax and hemp has been done for a trait "high/improved HMs uptake and accumulation" previously; the situation was just opposite—the selection was carried out for lines with low HMs (mainly Cd) seed content (Cieslinski et al. 1996; Li et al. 1997; Hocking and McLaughlin 2000; Eboh and Thomas 2005; Korkmaz et al. 2010) as related to hygienic aspect. Thus, the knowledge of genetic/phenotype differences in accumulation (and mainly transport to above-ground plant parts) and tolerance to particular HM elements is crucial for formulation of efficient phytoextraction technology. The candidate genotypes may be found/screened either within commercial varieties or germplasm resources. In fact, flax represents the only species within fibre crops with some available data on genotype screening for heavy metal tolerance/accumulation. Most of papers dealing with fibre crops and heavy metals reported experiments usually with one or two genotypes of particular crop, and thus, natural genetic variation in this trait remains still unknown.

The existence of genotype differences in the uptake and distribution of heavy metals by flax plants was confirmed unequivocally by many authors (Marquard et al. 1990; Marquard and Böhm 1992; Helal et al. 1991; Böhm et al. 1992;

Table 11.4 The summary of heavy metals uptake and accumulation by hemp (*Cannabis sativa* L.)

Hemp variety	Aim of study	HMs concentration in soil/solution (mg kg^{-1})	HMs concentration in plant/plant organs (mg kg^{-1} DW)						Reference
Not presented	Effect of N-fertilisation on selected microelements uptake	No data (pot experiment)		Fe	Mn	Zn	Cu	Mo	Jurkowska et al. (1990)
			Above-ground biomass:	750–882	576–896	127–143	6.10–7.31	0.11–018	
			Roots:	29–34	8–21	79–127	6.10–7. 01	0.10	
Bialobrzeskie	Cu effect on the Mn, Zn and Fe uptake ($CuSO_4$: 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 mg dm^{-3})	$FeSO_4.7H_2O$: 2–7.5 $MnSO_4.H_2O$: 0.25–0.6 $ZnSO_4.7H_2O$: 0.1–0.3 $Na_2MoO_4.2H_2O$: 0.1–0.3 (water cultures)		Fe	Mn	Zn	Cu		Jasiewicz (1991)
			Above-ground biomass:	150–270	50–100	60–80	5–30		
			Roots:	500–1,800	10–40	50–100	10–2,000		
			(approximate values read from graphs)						
Not presented	Effect of N-fertilisation and soil moisture content on selected microelements uptake	No data (pot experiment)		Fe	Mn	Zn	Cu	Mo	Jurkowska et al. (1992)
			Above-ground biomass:	98–393	118–481	83–136	5.37–7.69	0.36–0.53	
			Roots:	536–950	183–398	373–532	11.65–16.38	0.38–0.55	
Bialobrzeskie	Toxic effect of HMs on plant growth/yield; effect of liming and P-fertilisation on HMs phytoavailability	Cd: 1, +8 Cu: 7.8, +30 Ni: 8.4, +50 Pb: 31, +100 Zn: 83.1, +200 (pot experiment)	Plant organs not distinguished (above-ground + root biomass)						Gorlach and Gambuś (1992)
				Cd	Pb	Zn	Cu	Ni	
				0.017–0.105	0.199–0.354	1.17–7.69	0.159–0.249	0.065–0.995	
Bialobrzeskie	Effect of liming ($CaCO_3$) on phytoavailability of HMs	Cd: 1.0 Soil I pH = 4.6 Cu: 7.6 Soil II pH = 6.6 Ni: 8.4 (Ca-liming) Pb: 31.0; Zn: 83.1 (pot experiment)		Cd	Pb	Zn	Cu	Ni	Gorlach (1994)
			Above-ground: I	2.4	6.7	132	9.4	4.5	
			Biomass: II	1.0	5.5	28	5.4	1.4	
			Roots: I	11.0	9.0	386	10.9	9.2	
			Root: II	4.0	3.3	71	8.7	2.8	
Bialobrzeskie	On site experiment in polluted soil; accumulation of HMs and their effect on yield and quality of hemp biomass	Cu: 135 Pb: 66 Zn: 21 Cd: 0.3 (on site experiment)		Cd	Pb	Zn	Cu		Baraniecki and Mankowski (1995)
			Seed:	0.17	0.8	64.6	21.3		
			Stem:	0.11	2.0	11.6	9.7		
			Root:	0.32	6.0	21.5	41.7		

Bialobrzeskie, Beniko	On site experiment in polluted soil; accumulation of HMs and their effect on yield and quality of hemp biomass	Cu: 162–200 Pb: 67–70 Zn: 19–42 Cd: <0.5 (on site experiment)			Pb		Cu			Baraniecki et al. (1995)
			Seed:		0.95–1.00		19.8–20.5			
			Stem:		1.20–3.20		7.4–11.6			
			Fibre:		2.90–3.90		8.9–9.3			
Not presented	Effect of irrigation with sewage water on plant microelement content	Cu: 0.011 Zn: 0.173 (field experiment)			Mn	Zn	Cu			Labuda and Kaczor (1999)
			Whole plants:		18–33	35.5–46.0	2.5–10.5			
Beniko	Effect of sewage water on yield and microelement content of hemp	Cu: 0.11 Zn: 0.173 (pot experiment)		Fe	Mn	Zn	Cu			Wisniewski and Kolodziej (1999)
			Above-ground biomass:	69–131	6.83–9.13	41–56	0.92–2.46			
Bialobrzeskie, Beniko	On site experiment in polluted soil; accumulation of HMs and their effect on yield; two fertilisation variants	Cu: 51.69–183 Cd: 0.19–<0.5 Pb: 7.55–70 Zn: 14.37–46 (on site experiment)		Cd	Pb	Zn	Cu			Baraniecki et al. (2001)
			Seed:	0.02–0.13	0.55–2.40	54.60–76.50	0.80–27.80			
			Stem:	0.02–0.09	1.00–4.20	3.70–24.30	1.00–13.90			
Bialobrzeskie, Beniko	On site experiment in polluted soil; accumulation of HMs and their effect on yield	Cu: 82.24–183 Cd: 0.21–<0.5 Pb: 17.9–70 Zn: 14.37–46		Cd	Pb	Zn	Cu			Kozlowski et al. (2002)
			Seed:	0.03	1.4–1.5	55.1–55.3	2.2–23.3			
			Stem:	0.035–0.40	2.1–2.2	5.6–6.3	4.8–5.0			
JUSO 31	Effect of HMs on fibre quality; phytoremediation aspect	Cd: 102 Ni: 419 Pb: 454 (field trial; sewage sludge application)		Cd	Pb	Ni				Linger et al. (2002)
			Leaves:	2.96–3.94	21.65–23.20	63.46–63.83				
			Seeds:	1.03–1.19	1.69–1.98	24.79–33.24				
			Fibres:	0.79–0.85	3.78–3.97	6.32–7.42				
			Hurds:	0.76–0.78	2.60–2.94	9.99–12.96				
Kompolti	Conditioning of HM-contaminated river sediment; phytotoxicity tests of HMs; commercial use of contaminated biomass;	Sediment/ Reference soil Cd: 17/<2 Pb: 187/60 Ni: 174 Zn: 2,120/75 As: 31/10 Cr: 333/45	Sediment							Löser et al. (2002)
				Seeds	Young leaves	Fibres	Shives	Old leaves	Roots	
			Cd:	1.4	2.0	5.2	1.6	4.2	2.4	
			Zn:	410	1,300	1,100	230	2,600	480	
			Reference soil							
				Seeds	Young leaves	Fibres	Shives	Old leaves	Roots	
			Cd:	<0.1	0.1	<0.1	<0.1	0.1	0.1	
			Zn:	70	60	<20	<20	40	<20	

(continued)

Table 11.4 (continued)

Hemp variety	Aim of study	HMs concentration in soil/solution (mg kg^{-1})	HMs concentration in plant/plant organs (mg kg^{-1} DW)					Reference
	phytoremediation aspect	Cu: 208/25 Hg: <2/<2						
Fibranova	Hemp tolerance and ability to accumulate Cd, Ni and Cr	Cd: 26.6–82 Ni: 49.6–114.6 Cr: 117.7–138.8 (pot experiment; semi-natural conditions)		Cd	Ni	Cr		Citterio et al. (2003)
			Leaf:	0.3–58.8	2.7–31.4	1.2–1.8		
			Stem:	0.4–73.0	14.0–52.1	n.d.		
			Root:	0.6–1,368.2	3.4–321.8	2.3–6.9		
Not presented	Phytoextraction of Pb, Zn and Cd from soil increased by using chelators	Pb: 1,100 Zn: 800 Cd: 5.5 (pot experiment; semi-natural conditions, soil from industrial site)	Only above-ground tissue collected					Kos et al. (2003)
				Cd	Pb	Zn		
				0.17–0.76	10–220.58	90.36–105.07		
Not presented	Soil washing of Pb, Zn, and Cd and induced phytoextraction using chelators	Pb: 1,750 Zn: 1,300 Cd: 7.2 (pot experiment; soil from industrial site; pots with horizontal permeable barriers)	Only above-ground tissue collected					Kos a Leštan (2004)
				Cd	Pb	Zn		
				0.35–3.84	0.53–1,026.49	44.29–330.28		
Benico	Phytoextraction of heavy metals by hemp during anaerobic sewage sludge management in the non-industrial sites	Zn: 8.06–30.15 Cu: 1.56–5.69 Ni: 0.89–2.66 (pot experiment; greenhouse conditions)			Cu	Zn	Ni	Piotrowska-Cyplik and Czarnecki (2003a, b, 2005)
			Root:		1.23–4.63	8.42–36.74	0.32–2.56	
			Stem:		0.74–1.61	1.08–9.40	0.2–0.75	
			Leaf:		0.93–6.82	6.35–16.32	0.21–1.10	
Not presented	Effect of HMs on the yield; detoxification of polluted soil	Cd: 5–320 Cu: 20–1,280 Ni: 15–960 Pb: 30–1,920	Plant organs not distinguished					Antonkiewicz et al. (2004)
				Cd	Pb	Zn	Cu	Ni
				0.32–31.48	1.99–76.52	19.00–267.33	2.69–32.13	4.90–38.09

		Zn: 50–3,200 (pot experiment)									
Silistrinski	On site experiment on polluted soil; accumulation and organ distribution of HMs	On site experiment; distance 0.5 and 15 km from Metal Factory Cd: 2.5–12.2 Pb: 22.7–200.3 Cu: 13.9–95.7 Zn: 31.9–536.1		Cd	Pb	Zn	Cu				Angelova et al. (2004)
			Root:	0.35–1.03	3.8–38.2	15.5–66.8	4.9–7.2				
			Stem:	0.33–0.98	2.4–23.5	12.7–54.5	3.3–4.9				
			Leaves:	0.19–0.55	1.9–16.5	9.3–40.0	2.5–3.6				
			Flower:	0.41–1.22	4.5–44.8	18.3–78.6	6.9–10.2				
			Fibre:	0.15–0.40	2.1–6.3	1.3–19.9	1.3–1.8				
			Seeds:	0.34–1.00	1.0–7.6	17.8–73.5	5.9–8.9				
Fibranova	Cu localisation in hemp plants by electron microscopy and X-ray microanalysis	$CuSO_4$: 0.01, 0.1, 1, 2.5, 5, 7.5, 10 mM (hydroponic culture)	Stem: Cu not detected in primary bast fibres (Cu is probably excluded from the stem) Leaf: Cu preferentially accumulated in the upper leaf epidermal cells and leaf trichomes								Arru et al. (2004)
Not specified–fibre cultivar	Effect of different Cd concentrations on growth, Cd uptake and photosynthesis	Cd: 17 and 72 (pot experiment, greenhouse conditions)	Concentration of Cd reached a maximum after 24 days growing, than rapidly decline.								Linger et al. (2005)
			Root:			830–42					
			Stem:			87–20					
			Leaf:			68–15					
Carmagnola	Effect of arbuscular mycorrhiza on HM uptake and translocation	Total/Bioavailable Cd: 140/45 Ni: 120/35 Cr: 50–350/– (pot experiment; semi-natural conditions)		Cd	Ni	Cr					Citterio et al. (2005)
			Root:	163.9–217.2	4.3–122.5	1.0–47.5					
			Stem:	12–16.6	0.2–29.1	0.5–1.3					
			Leaf:	11.2–12.1	4.8–17.6	1.8–4.3					
			Seed:	7.7–7.8	2.3–17.1	0.1–0.2					
Not presented	Analysis of heavy metal content in leaf and seed	Not analysed; natural site in Africa/Nigeria	Only leafs and seeds were analysed:								Eboh and Thomas (2005)
				As	Cd	Cr	Fe	Ni	Pb	Hg	Mn
			Leaf:	13.6	4.4	17.41	24	10.40	1.58	19	28
			Seed:	7.48	2.4	15.2	7.8	6.8	0.42	6.16	102.5
Not presented	Monitoring of heavy metals and selected micronutrients in hempseed	Not analysed; natural sources from Turkey	Only seeds were analysed (μg kg^{-1}):								Korkmaz et al. (2010)
			Al	Ni	Co	Cd	Cu	Zn	Fe	Mn	B	Mo
			5–24	0.36–1.66	0.05–0.17	5–23	9–12	46–72	98–121	70–102	11–14	0.27–0.62

Gaudchau and Schneider 1996; Schneider et al. 1996; Schneider and Marquard 1996; Cieslinski et al. 1996; Li et al. 1997; Becher et al. 1997; Kokurin and Yagodin 1997; Grant et al. 2000; Hocking and McLaughlin 2000; Bjelkova et al. 2001; Lukipudis 2001). By comparing 20 flax genotypes, Böhm et al. (1992) found that only seven tested cvs responded sensitively to Cd content changes in the soil; all other genotypes behaved independently, thus the Cd concentration in the stem could not be simply derived from the soil Cd content. Gaudchau and Schneider (1996) and Schneider and Marquard (1996) reported significant genotype differences in Cd accumulation of linseed—cv. Marigold accumulated more Cd in the seeds, while cv. McGregor retained significantly more Cd in roots. Increasing of soil Cd concentration (0.12, 0.72 and 1.3 mg Cd kg^{-1} soil) surprisingly resulted in increased Cd transport to seeds and in decreased Cd retaining by roots. According to authors, great differences in Cd content in particular plant organs are predetermined by genetically based different distribution in plants (i.e. different translocation and accumulation in seeds). Thus, at concentration 0.12 mg Cd kg^{-1} soil, cv. McGregor retained 92 % uptaken Cd in root and stem and only 8 % was transported to the seeds, while in cv. Marigold there are quite different relations: 61 % uptaken Cd was retained by root and stem and 39 % was translocated to the seeds. Similar conclusions (cv. McGregor accumulates very low seed Cd concentrations) were done by Becher et al. (1997). Some authors (Helal et al. 1991; Cieslinski et al. 1996; Becher et al. 1997) supported the idea that genotype differences are a result of different Cd redistribution within shoots and not of Cd uptake and translocation from roots to shoots. Becher et al. (1997) stated that genotype differences were restricted to Cd and were no general phenomenon of supply of generative organs with micronutrients (e.g. Zn, Cu).

The most complex and detailed study of *L. usitatissimum* L. as related to Cd, covering 4-year field-simulated experiment, broad range of soil Cd concentrations (10 to 1,000 mg Cd kg^{-1} soil), both technological *L. usitatissimum* types—flax and linseed, ten commercial flax/linseed cultivars and four analysed plant organs has been recently published by Bjelková et al. (2011a, b). The most Cd was accumulated by roots, followed by shoots, while reproductive parts (capsules and seeds) played comparably smaller role. The increasing soil Cd concentration resulted in increasing Cd accumulation by roots, while transport to above-ground plant parts was progressively inhibited. Even high soil Cd concentrations (1,000 mg Cd kg^{-1} soil) had not dramatic negative effect on plant growth and development. Cultivar differences as well as the differences between both technological *Linum* types have been found in Cd accumulation (flax being better Cd accumulator than linseed). Nevertheless, the recorded variation between technological types and within cultivars was in multiples of Cd values (units of mg Cd kg^{-1} DW), not in orders of magnitude as needed for highly efficient phytoextraction. A significant year-to-year effect on plant growth/development resulting in high variation in Cd accumulation was observed. Flax cv. Jitka exhibited good transport of Cd from roots to above-ground parts, while flax cv. Merkur showed high retention of Cd in roots. Further, the contrasting cultivars in total Cd accumulation (high accumulating flax cv. Jitka versus low accumulating linseed cv. Jupiter) were selected.

Majority of published reports on Cd uptake by flax/linseed was dealing with natural (geogenic) or slightly increased soil Cd concentrations (usually less than 0.5 mg Cd kg^{-1} soil), which may be categorised as non Cd-contaminated soils (Cieslinski et al. 1996). Broadley et al. (2001) analysed phylogenetic variation in heavy metal accumulation (based on published data) and found flax ranked 22nd of 108 totally recorded plant species (wild species + crops) as related to relative shoot Cd content, and even 4th of 51 recorded crops/grasses/vegetables. This finding categorises flax/linseed in the upper border in above-ground Cd accumulation within up to date studied crops. Nevertheless, according to Baker (1981) classification, flax/linseed may be classified as related to Cd as "indicator" (i.e. internal Cd concentration reflects external Cd levels) not accumulator species. High soil Cd concentrations used in experiments of Bjelková et al. (2011a, b) were probably never published before—only Chakravarty and Srivastava (1997a, b) used 50– 2,000 μM Cd (= 5.62–224.82 mg Cd L^{-1} culture medium) in tissue culture medium for linseed. Such Cd concentrations do not occur in polluted agricultural soils, but they should answer the question of accumulation potential of flax/linseed for Cd and the effect of such toxic Cd concentrations on the plant growth and development. Broadley et al. (2001) also concluded that accumulation potential of particular plant species may be better demonstrated in a high metal environment than in a low metal environment. Even 1,000 mg Cd kg^{-1} soil did not result in severe damage of flax/linseed plants; nevertheless, it is evident that only a part of artificially added Cd belongs to bioavailable soil Cd fraction. In addtition, it is clear that—at Cd soil concentrations of hundreds of mg Cd kg^{-1} soil—substantial portion of the metal is blocked in the roots. To answer the questions of genetic variation in HMs accumulation and namely HMs distribution in plant organs, the experiments allowing growth/development of complete mature plants represent an optimum approach. Thus, pot experiments with soil or representative field trials may give rigorous results on behaviour of plants in real environment. Nevertheless, hydroponics and laboratory experiments (if well designed), may bring useful supplementary information. Here we provide several examples in flax/linseed. Bjelková et al. (2007) reported that 10 and 100 mmol L^{-1} concentrations of HMs (ten metal elements tested) resulted in lethality in all flax and linseed cultivars in a standard germination test. Similarly, Soudek et al. (2010) studied 23 flax/linseed cultivars as related to heavy metal toxicity based on seed germination test (=inhibition of root elongation). They have found a high diversity in the response of flax/linseed cvs to particular heavy metal elements. No cultivar exhibited total tolerance/resistance to a spectrum of toxic heavy metals, but there were specific interactions cv x metal element. In general, the heavy metal toxicity decreased in the following order: $As^{3+} \geq As^{5+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Cr^{6+} > Ni^{2+} > Pb^{2+} > Cr^{3+} > Zn^{2+}$. Linger et al. (2005) reported that Cd concentrations up to 72 mg kg^{-1} soil had no negative effect on hemp seeds germination.

Tissue culture experiments in vitro may help in quick screening of HMs tolerant or accumulating genotypes/lines on HMs-supplemented culture media (nevertheless, the recorded data should correspond to pot or field trials in order to reflect real situation). Chakravarty and Srivastava (1997a, b) first tested the effect of Cd stress (50–2,000 μM $CdCl_2$) on callus growth and plant regeneration in vitro in three

Indian linseed cultivars (Garima, Gaurav and Kiran). Cv. Kiran appeared to be least affected by the presence of Cd. Total protein and proline content increased due to Cd treatment in callus lines, but there were no differences between cultivars tested. The plantlets of cv. Garima tolerant to Cd^{2+} and Zn^{2+} were selected on medium with equimolar concentrations of both elements. Cd was retained mainly in roots (in cytoplasm), while Zn was translocated to shoot and accumulated in the cell wall. Peroxidase activity was lower in the Cd/Zn tolerant plantlets as compared to sensitive controls.

Tejklová et al. (2007) screened 23 flax and linseed varieties, registered in the Czech Republic (the same set as reported by Soudek et al. 2010), in vitro on a Cd-supplemented culture medium. Hypocotyl segments (5–10 mm) from aseptically germinated seedlings were cultured on MS medium (0.005 μM NAA, 1 μM BAP) with increasing concentrations of $Cd(NO_3)_2$—0 (control), 5, 10, 50 mg L^{-1} medium. After 7 weeks, the following parameters were recorded in proliferating multiple shoot culture: number of viable and necrotic explants, callus formation, number of buds, shoots and roots per explant and Cd-content in explants and agar medium. The investigation of selective Cd concentration showed that 5 and 10 mg L^{-1} $Cd(NO_3)_2$ caused only weak growth/regeneration reduction in vitro, while 50 mg was completely lethal. Thus, sublethal concentration 40 mg L^{-1} Cd $(NO_3)_2$ was used in further experiments. Significant differences in Cd tolerance (based on explant viability and growth parameters) were recorded among flax/linseed varieties (the most tolerant ones: Ilona, Lola, Atalante, Jitka; the most sensitive ones: Tábor, Marylin, Merkur, Super). AAS analysis showed the content of Cd differs both among varieties (Jitka, Biltstar, Lola—maximum accumulation; Atalante, Ilona—minimum accumulation) and among explant parts (callus with buds absorbed more Cd than shoots). More recently (Smýkalová et al. 2010), these data were extended also to Zn^{2+}. 25 flax/linseed varieties were screened on sublethal Cd (19 mg L^{-1} Cd^{2+}) and Zn (33 mg L^{-1} Zn^{2+}) concentrations using two regeneration approaches from cultured hypocotyl segments. Both growth parameters in vitro (number of organogenic points; dry matter) and Cd/Zn accumulation were evaluated. Five flax (Ilona, Jitka, Merkur, Tábor, Venica) and three linseed cvs (Atalante, Biltstar, Lola) were selected as the best Cd/Zn accumulating ones and this set was further studied in detail. Flax variety Jitka showed superior Cd tolerance (= explants stayed green onto Cd medium, they produced buds, shoots and roots) and highest Cd/Zn accumulation capacity. In contrast, cv. Merkur while being tolerant based on growth parameters in vitro, showed the lowest Cd accumulation. Thus, two different mechanisms of Cd tolerance were proposed in the group of tolerant cvs (1) the first type is connected with a restricted uptake of Cd ions and low Cd accumulation in tissues, (2) the second represents easy uptake of Cd ions and their high accumulation in tissues, where Cd ions are detoxified and deposited in cytoplasm or vacuole (thus protecting cells and tissues from Cd toxicity). The main output of extensive in vitro and field experiments of our laboratory is that flax cv. Jitka represents a genotype with high tolerance to Cd/Zn with good root-to-shoot transport of HMs and high accumulation of HMs in above-ground biomass. The determination of contrasting flax genotypes as related to Cd tolerance/accumulation

(Tejklová et al. 2007; Smýkalová et al. 2010; Bjelková et al. 2011a,b) enabled the study of proteome changes as well as the phytochelatin induction by Cd stress, in order to better understand mechanisms of HMs tolerance in flax/linseed. Hradilová et al. (2010) compared proteomic changes of two contrasting genotypes (Jitka—tolerant/high Cd accumulation; Tábor—low tolerant to sensitive/high Cd accumulation) in suspension cultures upon Cd exposure (10, 50 and 100 μM Cd $(NO_3)_2$). Significant changes in the expression of 14 proteins (2-DE followed by MALDI-TOF/TOF MS) were detected (proteins related to disease/defence, metabolism, protein destination and storage, signal transduction, energy and cell structure). Two proteins, ferritin and glutamine synthetase (a key enzyme in glutathione biosynthesis) were only up-regulated by Cd in cv. Jitka, indicating that Cd tolerance mechanisms in this cultivar may include maintenance of low Cd levels at sensitive sites by ferritin and low-molecular weight thiol peptides binding Cd. Vrbová et al. (2009) studied phytochelatins (PCs) induction by Cd treatment in the same experimental design of suspension cultures of cv. Jitka and Tábor. Samples were collected after 3, 12, 24 and 48 h after Cd treatment and analysed using the adsorptive transfer stripping (AdTS) linear sweep voltammetry at HMDE. Results show remarkable Cd concentration- and time of cultivation-dependent changes in the PCs levels. In both cultivars, 10 μM $Cd(NO_3)_2$ appeared to induce PC accumulation without affecting the cells viability. Lower PC levels observed for cv. Tábor cultivated in the presence of 50 μM $Cd(NO_3)_2$ for 3–24 h can be attributed to inhibition of cell growth and/or dying of certain population of the cells at this Cd level, while the intense signal observed after 48 h to accumulation of high PC levels in surviving cells. In the more tolerant cv. Jitka, much lower PC levels were observed during cultivation in 50 μM $Cd(NO_3)_2$, which may be in agreement with the speculation (Fojta et al. 2006) that extremely high amounts of the induced PCs may contribute to the heavy metal lethality. The prevailing lethal effect of 100 μM $Cd(NO_3)_2$, in agreement with no or only relative small increase of the induced PC amounts, was detected in both flax cvs. The results indicate that the upper limit of Cd tolerance in *L. usitatissimum* cell suspension is 50 μM (depending also on cultivar) and higher concentrations cause toxicity to the cells. PCs changes were significantly enhanced with increasing concentrations of Cd in the medium. Najmanova et al. (2012) studied Cd-induced PCs induction and intracellular Cd localisation in 12-days-old linseed seedlings (line AGT 952) grown on agar medium supplemented with 50–1,000 μM $CdCl_2$. Majority of accumulated Cd was retained in roots, which showed reduced elongation as related to Cd concentration in the medium. Several classes of PCs (PC2, PC3, PC4, PC5) were detected by HPLC in seedlings organs. Regardless the treatment, PC3 was dominant in all organs and PC2 was restricted to cotyledons. Larger PC4 and PC5 accumulated only in roots grown at 500 μM Cd. In roots, the majority of Cd was stored in $\geq$9 kDa complex corresponding by molecular mass to stable high-molecular weight (HMW) CdS–PC complexes of yeasts and plants. Data of both studies demonstrate that response of flax to Cd involves phytochelatins and suggest the deposition of Cd in HMW complex in root should be considered as a mechanism preventing Cd translocation to shoot. Nevertheless, there is necessary to study these processes in complete flax plants differing in HMs (Cd) uptake,

transport and accumulation in order to find biochemical/molecular markers connected with low/high retention of HMs in roots and their easy/hard transport to above-ground plant parts, thus reflecting both hygienic and phytoremediation aspect, respectively. As the laboratory analyses of HMs content are very expensive, such markers may speed the selection of genotypes of interest (Penner et al. 1995). Existence of variation in the uptake, translocation and accumulation of heavy metals by flax is a prerequisite for selection and further breeding both low- and high-accumulating genotypes.

The field screening of *Linum* germplasm resources (AGRITEC Flax Collection; location with natural background of heavy metals in soil) for a trait Cd/Pb/Zn accumulation has not been finished yet (in 2010–2011, ca. 350 accessions analysed—Bjelková, unpublished results)—the variation exists, nevertheless it is not large enough to select hyperaccumulating genotypes (Cd stem range: 0.05–3.49 mg Cd kg^{-1} DW; Pb stem range: 0.15–2.72 mg Pb kg^{-1} DW; Cd seed range: 0.34–2.22 mg Cd kg^{-1} DW; Pb seed range: 0.23–4.62 mg Pb kg^{-1} DW; Zn stem range: 1.46–96.42 mg Zn kg^{-1} DW; Zn seed range: 40.82–84.23 mg Zn kg^{-1} DW). In contrast to flax/linseed, there is no evidence in available literature on genetic differences in heavy metal uptake and accumulation by hemp plants as majority of papers were dealing only with one variety or even the experimental material was not described (Table 11.4). The following hemp cvs were studied as related to HMs in the course of last 20 years: Bialobrzeskie, Beniko, Juso 31, Kompolti, Fibranova, Silistrinski, Carmagnola (Jasiewicz 1991; Jurkowska et al. 1990; Gorlach and Gambuś 1992; Gorlach 1994; Baraniecki and Mankowski 1995; Baraniecki et al. 1995, 2001; Kozlowski et al. 2002; Linger et al. 2002; Löser et al. 2002; Citterio et al. 2003, 2005; Angelova et al. 2004). Only Baraniecki et al. (1995, 2001) and Kozlowski et al. (2002) used in on site experiments two hemp varieties for comparison (cvs Beniko and Bialobrzeskie). Despite the absence of statistical evaluation, there were negligible differences between these two varieties in the accumulation of Cu and Pb in the seeds, stems and fibre—the only exception was the accumulation of Pb in stems (cv. Beniko accumulated more than 2.5 times more Pb than cv. Bialobrzeskie; 3.20 versus 1.20 mg Pb kg^{-1} DW) (Baraniecki et al. 1995). However, later experiments of the same group showed more or less similar accumulation of Cu, Pb, Zn and Cd by both cvs with dominating year-to-year effect or the fluctuating data did not show any clear tendency (Baraniecki et al. 2001; Kozlowski et al. 2002). Our experiments (Bjelková et al. 2005 and unpublished results) designed to compare four hemp cvs (Beniko, Glera, Juso, Silesia) showed minimal differences in Cd uptake in geogenic Cd concentrations.

The analysis of published data results in two conclusions (1) The genetic variation for HMs accumulation in above-ground biomass in flax/linseed is limited (even in germplasm resources) and thus, the original expectations have not been fulfilled (i.e. finding genotypes with the level of HMs accumulation close to wild hyperaccumulators); (2) There are no data in literature on genetic variation for HMs accumulation in hemp—the screening of genotypes/lines/cultivars must be still done to obtain first view of the problem. It is a question, if would be reasonable to start conventional breeding for HMs tolerance and accumulation and what would

be a cost and success of such approach. Thus, the more logical next step will be to engineer transgenic fibre crops with genes responsible for HMs transport or accumulation from wild plant species (hyperaccumulators) or from organisms other than plants (e.g. mammalian metallothionein—Vrbová et al. 2012).

11.5.2 Organ Distribution of Heavy Metals

There are two opposite requests for HMs accumulation (1) hygienic or food aspect prefers high retention of toxic metals in the roots with minimum transport to edible parts (seeds), while (2) phytoremediation aspect stresses the easy transport of HMs to above-ground biomass. Studies of heavy metal accumulation in flax plants mostly exhibited maximum concentrations in roots. Common concentration gradient for Cd is following: root > shoot > capsule ≥ seed (Böhm et al. 1992; Böhm and Marquard 1993a, b; Baraniecki et al. 2001; Jiao et al. 2004; Angelova et al. 2004). Nevertheless, the concentrations overcoming hygienic limit in seeds (0.3 mg Cd kg^{-1} DW) were very often observed, sometimes exceeding also stem concentration (Gaudchau and Marquard 1990; Heyn and Janssen 1991). Grabowska and Baraniecki (1997) and Baraniecki et al. (2001) found the highest Pb and Cu concentrations in capsules and Zn in seeds. Grzebisz et al. (1997a) found in Pb following concentration gradient: root > shoot > seed > capsule, in Cu: capsule > root > seed > shoot (see also Mankowski et al. 1994). In contrast, Straczynski and Andruszczak (1996) reported the highest accumulation of Pb in capsules and Cd in shoot of flax irrespective of various degree of Cu and Pb pollution of tested soils. As related to produced biomass of flax/linseed, the total Cd accumulation (concentration × biomass) in particular organs may exhibit following gradients: shoot > seed > capsule = root (Böhm and Marquard 1993a, b; Schneider et al. 1996), shoot > root > seed > capsule (Cieslinski et al. 1996; Bjelkova et al. 2001). Artificial increase of soil Cd (above natural levels) resulted in higher Cd accumulation in roots (Bjelkova et al. 2001) and in the shift of distribution between plant parts—seeds accumulated more Cd, while shoots less Cd (Böhm and Marquard 1993a, b). Hocking and McLaughlin (2000) found—by comparison of Cd distribution in several crops, namely linseed, canola, Indian mustard, two lupin species and wheat—that physiological mechanisms preventing Cd translocation from the fruit to seeds are much less effective in linseed than in all other studied crops. Also Becher et al. (1997) considered differences in Cd translocation from fruit to seeds of linseed as the main feature of genotype variability of Cd accumulation in the seed.

As mentioned before, majority of published reports on Cd uptake by flax/linseed was dealing with natural (geogenic) or slightly increased soil Cd concentrations (usually less than 0.5 mg Cd kg^{-1} soil), which may be categorised as non Cd-contaminated soils. Unfortunately, in such soil (which is probably true also for other metal elements and other culture crops) the varietal differences in tolerance/accumulation as well as differences in organ distribution of HMs are not clearly evident. These differences are better visible on soils with HMs concentrations

elevated in orders of magnitude (10 times, 100 times higher; Broadley et al. 2001; Bjelková et al. 2011a). Thus, it is interesting to know, what is the behaviour of flax and hemp plants in such "drastic", more or less non-physiological conditions? It means—are there any negative effects on plant growth and development, yield and quality of final harvestable product (fibre, seeds)? Gaudchau and Marquard (1990) did not find any negative effect of increased soil Cd concentration on the yield of seeds, oil content and fatty acid composition of two linseed varieties. In general, the natural content of Cd (or other heavy metals) did not influence growth and development of flax plants as well as their yield parameters (Moraghan 1993; Mankowski et al. 1994; Baraniecki et al. 1995, 2001; Jankauskiene 1998). Sligthly elevated concentrations of some heavy metals had even stimulatory effect; only significantly increased soil concentrations led to yield reduction, primarily in seeds, less in the stem (Moraghan 1993; Mankowski et al. 1994; Jankauskiene 1998) or even resulted in plant death (Linger et al. 2005). Grzebisz et al. (1997b) also reported minor effect of heavy metals on flax yield in the soils polluted by industrial activity (copper smelting), and the key significance was considered to sufficient nutrient supplementation and proper agrotechnology. Jankauskiene (1998)—based on the field experiments with fibre flax—did not find yield depression up to the following soil levels of heavy metals (mg kg^{-1} soil): Cr—24, Cd—1, Pb—16, Ni—14, Cu—8, Mn—200.

Our recent data (Bjelková et al. 2011a) showed that even very high Cd concentrations (1,000 mg $Cd(NO_3)_2$ kg^{-1} soil) did not have visible effect on flax/linseed plants growth and development. The soil Cd concentrations over 100 mg Cd $(NO_3)_2$ kg^{-1} soil resulted in dramatic increase of Cd retention in roots and very slow Cd transport to above-ground organs. Such elevated Cd soil concentrations helped to distinguish between flax/linseed cvs as related to distribution of Cd from root to shoot (or above-ground plant parts) (e.g. cv. Jitka—low Cd retention in root, high root-to-shoot transport; cv. Escalina—high Cd retention in root, low root-to-shoot transport). Such contrasting models may contribute to understanding of mechanisms HMs tolerance and transport by searching for specific markers—PCs, HMW complexes with HMs (Vrbová et al. 2009; Najmanova et al. 2012). Up to date results show that flax/linseed is able to tolerate elevated concentrations of some heavy metals in soil without evident yield depression or decrease of quality of harvested product—this fact has a crucial significance for further industrial processing of harvested biomass (stem—fibre, seed—oil).

The distribution/accumulation of HMs in hemp organs is summarised in Table 11.4. The early studies distinguished only between roots and above-ground biomass (Jurkowska et al. 1990, 1992; Jasiewicz 1991; Gorlach and Gambuś 1992; Gorlach 1994); as compared to flax early studies, more metal elements were studied in hemp, namely Fe, Mn, Zn, Cu, Mo, Cd, Pb, Ni and Cr. The content of heavy metals in roots and above-ground biomass was determined as influenced by N-, P- or Cu fertilisation and liming. In general, roots retained usually more Fe, Mn, Zn, Ni, Cu, Cd and Pb than above-ground biomass, while concentration of Mo was on similar level. Later on, Baraniecki et al. (2001) and Kozlowski et al. (2002) compared stem and seed concentrations at on site experiments and they found

similar values for Cd and Pb, while Cu and Zn were more translocated to seeds. Löser et al. (2002) used hemp for model remediation of river sediment highly polluted with HMs (e.g. Cd 17 mg kg^{-1} DW, Zn 2120 mg kg^{-1} DW) versus reference soil (e.g. Cd <2 mg kg^{-1} DW, Zn 75 mg kg^{-1} DW)—they studied phytotoxicity of HMs on hemp growth and also accumulation of selected metals in plant organs and hemp raw materials (fibre, shives) as related to further industrial processing. There were practically no differences in Cd accumulation in plant organs on reference soil, while Cd accumulation (mg kg^{-1} DW) from river sediment increased in the following order: seeds (1.4) < shives (1.6) < young leaves (2.0) < roots (2.4) < old leaves (4.2) < fibres (5.2). Hemp grown on (watered) sediment contained about 40 times more Cd, Zn and Ni than hemp cultivated on reference soil. The inhibition of hemp plants growth accompanied by some morphological/physiological abnormalities was considered to be a result of synergistic action of a low pH and several mobile toxic metals (especially Cd, Zn and Ni; Zn content of old leaves was even higher than in the settled sediment; Table 11.4). The high HMs (Cd) contamination of hemp fibre for further industrial processing is discussed below (Chap. 6). Linger et al. (2002) compared HMs accumulation and fibre quality of hemp (cv. JUSO 31) grown on HMs-polluted soil (application of sewage sludge with 102 mg Cd kg^{-1} DW, 419 mg Ni kg^{-1} DW and 454 mg Pb kg^{-1} DW). Leaves contained more HMs than seeds; nevertheless, fibres accumulated less Cd than reported by Löser et al. (2002)—the reason may be the lower Cd uptake from soil (limited bioavailability) in the field experiment versus hydroponics (easy uptake of mobile toxic metals). Linger et al. (2005) studied hemp growth, Cd uptake and photosynthesis parameters by hemp in pot experiment with two levels of artificial Cd contamination ($CdSO_4$): 17 and 72 mg Cd kg^{-1} soil. Cd concentrations up to 72 mg kg^{-1} soil had no negative effect on hemp seeds germination. Fresh mass and Cd content in plant organs was measured weekly for 7 weeks and then at the end of vegetation (133 days after sowing). The roots (plants grown on 17 mg Cd kg^{-1} DW) always accumulated the highest Cd concentrations with a maximum of 830 mg Cd kg^{-1} DW after 24 days, and then began to decline with plant growth. Stems and leaves accumulated Cd to a much lesser extent; the highest determined values were 87 and 68 mg Cd kg^{-1} DW in stem and leaves, respectively. At the end of vegetation period (133 days), the means were 42 mg Cd kg^{-1} DW for roots, 20 mg Cd kg^{-1} DW for stems and 15 mg Cd kg^{-1} DW for leaves. Plants grown on 72 mg Cd kg^{-1} DW displayed a very strong growth inhibition, and most plants died 4–5 weeks after sowing (only one plant survived this Cd treatment until 80 days within negligible biomass). In this study, hemp roots demonstrated a strong resistance to HMs (Cd) and have shown a somewhat "hyperaccumulator-like" potential (= more than 100 mg Cd kg^{-1} DW); however, this seemed to depend on the plant development stage (the ability of juvenile roots to produce phytochelatins and detoxify Cd; the loss of this ability in older roots). According to authors, the growth inhibition by high Cd concentrations may be connected with a strong difference between the stem/leaf and root meristems in Cd sensitivity/tolerance as well as with inhibition of photosynthesis. Angelova et al. (2004) recorded decreasing trend in Pb, Cu, Zn and Cd accumulation in hemp organs: flowers

(probably inflorescence) > roots > stems > leaves > seeds. Surprisingly high HMs content in flowers cannot be compared to other data as this is the only report on HMs content in hemp flowers (inflorescence) in literature. Citterio et al. (2005) studied HMs (Cd, Ni, Cr(VI)) accumulation changes caused by artificial mycorrhization (fungus *Glomus mosseae*) of hemp plants. Plants with artificially increased Cd, Ni and Cr concentrations accumulated most metal in the roots. However, in such soil, mycorrhization significantly enhanced the translocation of all three metal elements from root to shoot.

Similarly as in flax, it is difficult to make a generalisation for organ distribution of HMs within hemp plant, namely due to difficult comparability of particular reports (various methodology, namely hydroponics x pot experiment x field trial; various starting concentrations of HMs in soil/solution; various genetic materials used in experiments; various treatments to affect bioavailability of HMs, etc.). As related to hemp, it is also problematic to make conclusions from greenhouse pot experiments if the mature hemp plant normally reaches 2–4 m height in the field conditions. In such case, the field experiment represents the most objective approach. Despite of some contradicting data (Table 11.4) we may conclude the hemp plant tolerates and accumulates a spectrum of toxic metals without dramatic changes of growth and development when grown on medium concentrations of particular HMs. However, substantially increased contamination (e.g. 72 mg Cd kg^{-1} soil; Linger et al. 2005) results in irreversible damage of meristems, inhibition of photosynthesis and thus in growth retardation or plant death. In such comparison, the flax/linseed seems to be more tolerant (Bjelková et al. 2011a, b).

11.5.3 Agrotechnological Treatments to Improve Heavy Metal Phytoextraction

The series of studies have been carried out during last 25 years in Germany, Poland, USA, Canada and Australia, which evaluated an effect of soil and climatic factors on accumulation of heavy metals (mainly Cd) by flax plants. Some of the studies concentrated on simple collection of data from various locations and various soil types of certain country (sample analysis from farms; Germany, Australia) without active treatments of agrotechnology (Klein and Weigert 1987; Marquard et al. 1990; Li et al. 1997; Hocking and McLaughlin 2000), others evaluated an effect of P, N, Zn fertilisation, pH changes or artificial increase of total Cd or Zn in the soil on the uptake and accumulation of Cd or Zn in the seed and other plant parts. Experiments were realised both in the field conditions and in the greenhouse or growth chamber with collected defined soil samples. Extensive investigation (490 seed samples) of seed contamination of linseed by heavy metals in the whole region of Germany (Klein and Weigert 1987) brought first information on the seed content of essential as well as toxic metal elements (Table 11.5). Only several further studies followed accumulation of broader spectrum of metal elements in flax

Table 11.5 Content of metal elements in the seed of linseed (modified from Klein and Weigert 1987)

Metal element	Seed content (mg kg^{-1} DW)	Range (mg kg^{-1} DW)
Lead (Pb)	0.216	0.005–7.560
Cadmium (Cd)	0.353	0.005–1.330
Mercury (Hg)	0.013	0.0005–0.110
Arsenic (As)	0.044	0.005–0.410
Chromium (Cr)	0.211	0.050–0.542
Copper (Cu)	11.184	4.000–18.300
Manganese (Mn)	[a]	16.000–27.800
Nickel (Ni)	[a]	0.720–2.200
Selenium (Se)	0.250	0.025–1.110
Zinc (Zn)	52.380	32.600–151.600

[a]Mean value not presented due to low number of measurements ($n = 6$)

(Straczynski and Andruszczak 1996; Jankauskiene 1998; Belopuhov et al. 2001), majority of reports concentrated on Cd, less on Pb, Zn or Cu. Thus, the most consistent data are available in Cd and also the attempts to make certain generalisation/conclusions in this review have been derived mainly from Cd results.

Environment, as influenced by site and year, had the greatest effect on heavy metal concentrations in seed and tissue in the field experiments. The concentration of bioavailable soil Cd (in contrast to total Cd) is the key factor for Cd uptake by flax and in the certain concentration interval it may be proportional to accumulated Cd (Moraghan 1993). This factor mostly dominated over other factors, including significant genotype differences in Cd uptake and accumulation. The content of bioavailable metal element may represent only very small fraction of its total content—e.g. in the case of Pb in industrially polluted soil it was 1.7 % (Grzebisz et al. 1997a). Growing of flax in naturally metal–rich soils resulted in several times higher accumulation as compared to sites with lower heavy metal content (Schneider and Marquard 1996; Cieslinski et al. 1996; Grant et al. 2000). Cd concentration in the seed of the same linseed line/variety several times differed (up to sixfold) between various locations (Marquard et al. 1990; Schneider and Marquard 1996; Cieslinski et al. 1996; Grant et al. 2000). Numerous experiments used natural (geogenic) Cd soil concentrations (ca. 0.1–0.5 mg Cd kg^{-1} soil), artificial increase of soil Cd concentration led always to significant increase (up to 20-fold) of Cd content in flax tissues (Gaudchau and Marquard 1990; Heyn and Janssen 1991; Böhm et al. 1992; Böhm and Marquard 1993a, b; Moraghan 1993; Bjelkova et al. 2001). Cd accumulation in the seed sometimes overcame Cd soil concentration, e.g. by one order of magnitude larger (Heyn and Janssen 1991). Increased accumulation was mostly found on soils rich in nutrients, resulting at the same time in higher yields (Marquard et al. 1990). Accumulation of Cd in the seed was strongly related to the seed yield. Concentration and accumulation of Cd increased with increasing seed yield, both across soil types and across treatments

within a soil type (Grant and Bailey 1997). In contrast, accumulation of Zn was unrelated to seed yield. Several papers described competition between Cd and Zn uptake by flax plants (Moraghan 1993; Grant and Bailey 1997; Chakravarty and Srivastava 1997a). Zn and Cd are chemically similar and may compete for binding sites in the soil system and for uptake sites in the plant. At equimolar concentrations of Cd and Zn, Zn outcompetes Cd due to interactive ion uptake, resulting in reduction of Cd toxicity. Thus, based on this Cd–Zn antagonism or competitive effect, the Cd uptake may be manipulated through Zn soil bioavailability (soil-applied Zn may reduce Cd concentration in flax seed—hygienic aspect; decreased Zn soil availability may increase Cd uptake and translocation—phytoremediation aspect).

Nitrogen fertilisation elevated Cd concentration in flax seed and tissue in sites with naturally higher Cd soil content, but not in soils with low Cd backgrounds (Grant et al. 2000). As N fertiliser did not contain Cd, the increase in Cd concentration may be explained by the effect of fertiliser on soil chemistry and/or impacts on plant growth—N fertilisation may increase the bioavailability of Cd by increasing ionic strength of the soil solution or decreasing soil pH. N application may also increase root growth and plant vigour, which could increase the ability of the crop to access and accumulate Cd. Zn concentration in the seed and tissue decreased with N application (Grant et al. 2000). Phosphorus fertilisation (monoammonium phosphate containing Cd contamination) seems to increase Cd concentration and accumulation and to decrease Zn concentration in flax seed (Grant and Bailey 1997). Nevertheless, the results with P fertilisers later presented by the same group (Grant et al. 2000) were a little bit contradicting, namely that effects of N and P fertilisation on Cd concentration in flax seed were minor. Also Moraghan (1993) reported minimum effect of P fertilisation on Cd uptake by linseed. N, P and Cu ($CuSO_4$) fertilisation affected the bioavailability of several HM elements (Fe, Mn, Zn, Cu and Mo) also in hemp (Jurkowska et al. 1990, 1992; Jasiewicz 1991; Gorlach and Gambuś 1992).

A specific type of fertilisation is using sewage sludge, which not only is rich in organic as well as inorganic compounds, but which also brings to the soil toxic heavy metals. If well applied, sewage sludge may increase not only the biomass yield on the one side but also HMs accumulation on the other side as demonstrated both in flax and hemp (Piotrowska-Cyplik and Czarnecki 2003a, b, 2005; Bjelková et al. 2011b; Bjelková 2011). However, heavily polluted sediments, e.g. river sediments may results in severe HMs plant contamination (Löser et al. 2002).

The important component of HMs bioavailability in soil are chelating compounds (EDTA, DTPA, HEDTA, CDTA, NTA and citric acid), which increase the mobility or solubility of metal element binded on organic matter and thus its better phytoextraction. Kos et al. (2003) studied the effect of industrial soil (5.5 mg Cd kg^{-1}) on HMs uptake by hemp plants in above-ground biomass. The application of 5 mmol kg^{-1} EDDS (EDTA) resulted in significant increase of Cd (5.8 %) and Zn (16 %), in case of Pb the concentration was increased 22-fold as compared to control without EDTA.

In general, lower values of soil pH stimulate higher heavy metal uptake; nevertheless, published results in flax are not always unequivocal, sometimes even contradicting. Gaudchau and Marquard (1990) found small increase of Cd accumulation in the stem and seeds of linseed after CaO application. Böhm and Marquard (1993a, b) reported that liming of soils with natural Cd content did not result in evident decrease of Cd uptake. In contrast, liming of soils with artificially elevated Cd content explicitly decreased Cd uptake and Cd content in roots, stems and capsules; only seeds behaved indifferently. Heyn and Janssen (1991) proved an effect of liming (neutral soil pH recommended) on decreased Cd uptake by linseed plants—more markedly in seeds than in the stem. Schubert (1992) found higher Cd uptake in seeds in alluvial soil with pH = 7.2, high clay fraction (63 %) and higher organic matter content (3.3 %) as compared to brown soil with pH = 6.7, 12 % of clay and 0.4 % organic matter. Cd uptake by flax roots in hydroponics increased in pH range 4.0–6.0 (pH = 6.0 being optimum), pH = 7.0 induced drop in Cd uptake. Increasing $CaSO_4$ concentration in hydroponics resulted in decreased Cd uptake by flax roots (Schubert 1992). Lukipudis (2001) tested three cvs of fibre flax in the region of heavy polluted soils (Zlatica-Pirdop valley, Bulgaria)—decreasing soil pH (pH 6.1, 6.0, 5.9, 5.5, 5.0 and 4.8) led to increasing uptake of Cu, Cd and Pb and their increased accumulation in the fibre and seed. Application of $CaCO_3$ (change of soil pH from 4.6 to 6.6) decreased the bioavailability of Cd, Cu, Ni, Pb and Zn in hemp (Gorlach 1994). The year-to-year effect is represented mainly by the level of precipitation. Higher soil water content may increase the mobility of Cd in the soil and the transport flux of Cd through the plant; thus, enhanced moisture may increase ability of flax to accumulate Cd and translocate it from the tissue to the seed (Grant et al. 2000; Bjelková et al. 2011a, b). Based on above-mentioned data it is evident that heavy metal distribution is considerably affected by experimental conditions, namely soil properties, as well as genotypes used. Thus, properly designed agrotechnology may either stimulate or inhibit uptake of HMs by flax and hemp.

11.5.4 On Site Studies

Despite the minimum scientific knowledge on heavy metals behaviour in flax and hemp crops in the early nineties of the last century, on site pilot studies started in Europe, particularly in Poland—the reason was the long-lasting tradition in the breeding and growing of flax and hemp in this country and also the search for new roles of these crops both in agriculture and in industry in the end of the century. In addition, phytoremediation technology emerged at this time as new approach to clean environment. Later, on-site studies were extended to Bulgaria (Lukipudis 2001), Slovenia (Kos and Leštan 2004), Germany and the Czech Republic (Tlustoš et al. 2006) also thanks to locations historically contaminated by heavy industry.

At the same time, the flax field trials in Canada and Australia were conducted from the hygienic (not phytoextraction) point of view.

Grzebisz et al. (1997a) studied Pb and Cu accumulation by hemp, flax, rapeseed and cereals in the Legnice-Glogow Industrial Region with industrial pollution of 9,800 mg Cu kg^{-1} soil and 2,200 mg Pb kg^{-1} soil (Copper Smelters Factory). Pb was accumulated with studied plants in the decreasing order: rapeseed > hemp > cereals > flax. Estimated phytoextraction potential of hemp was 141 g Pb ha^{-1} and of flax 39 g Pb ha^{-1}. Cu was accumulated with the following order: hemp > rapeseed > cereals > flax, with estimated phytoextraction potential of hemp 377 g Cu ha^{-1} and of flax of 54 g Cu ha^{-1}. The important fact was the yield of above-ground biomass of both fibre crops was not decreased as compared to non-contaminated sites. The results in hemp were compared with data obtained in the same region, but another locations with different HMs pollution (Grzebisz et al. 1997b).

Grabowska and Baraniecki (1997) and Baraniecki et al. (2001) working in the same industrially polluted region (locations Biechov and Žukowice) studied varietal differences and the effect of N-fertilisation (60 and 120 kg N ha^{-1}) on HMs accumulation (Cu, Pb, Zn and Cd) in hemp (cvs Beniko and Bialobrzeskie) and flax (Wiko, Nike and Alba). There were no differences in Cd accumulation between cvs and plant organs; in contrast, Pb was more accumulated in the stem as compared to the seed, while Cu was more accumulated in the seed. Higher N-fertilisation dose did not confirm expected increase in HMs accumulation; in addition the yield of above-ground biomass was higher in polluted soil. Straczynski and Andruszczak (1996) studied in the same Polish location the accumulation of Cu, Pb, Zn, Mn, Cd, Ni and Cr into seeds, leaves and stem of hemp cv. Bialobrzeskie. The Cu and Pb content in seeds, leaves and stems positively correlated with the concentrations of particular elements in the soil. Seeds concentrated maximum content of Ni. Ni, Cr, Cd and Mn content was comparable to contents recorded in non-contaminated location.

Angelova et al. (2004) studied bioremediation potential of fibre crops (hemp, flax, cotton) in polluted area in Bulgaria. The experimental plots were situated at different distances (0.5 and 15 km) from the source of pollution—the Non-Ferrous-Metal Works (MFMW) near Plovdiv. The content of studied heavy metals (Cd, Pb, Cu and Zn) decreased with the distance from the source of pollution. The highest concentration of studied HMs were found in inflorescence (flower) and the lowest in fibre with usual trend: flower (inflorescence) > root > stem > leaves > seed > fibre. Linger et al. (2002) studied on site accumulation of HMs by hemp (cv. JUSO 31) in Hagen (Nordhein–Westfalen, Germany). The field was fertilised with sewage sludge containing 102 mg Cd kg^{-1}, 419 mg Ni kg^{-1} and 454 mg Cd kg^{-1}. The HMs were accumulated with decreasing trend: Ni > Pb > Cd with the highest concentration in leaves. Cd was accumulated 26-fold less than Ni. Accumulation trend for particular elements was as follows—Ni: leaves > seed > hurds > fibres; Pb: leaves > fibres > hurds > seed; Cd: leaves > seed > fibres > hurds. The hemp plants accumulated 126 g Cd ha^{-1} per vegetation period.

The several examples of on site studies mainly show that results of greenhouse pot experiments (or even hydroponic experiments) very often do not correspond to field data (very complex environment). Nevertheless, these limited number of on-site studies with flax and hemp may put the more realistic view on the abilities of studied crops and help to solve the question, how the phytoextraction technology may be efficiently connected with practical agricultural production?

11.5.5 Time Needed for Cleaning Heavy Metal-Polluted Soils

The key point for utilisation of flax/linseed and hemp for HMs phytoextraction is total absorption of metal element from square unit during vegetation period. Probably first estimation of time needed to clean soil contaminated by Cd in fibre crops, namely in flax was reported by Böhm et al. (1992). The authors started with value 3 mg Cd kg^{-1} soil (after application of sewage sludge) in the upper layer of soil, which represents in total 9 kg Cd ha^{-1}. If the seed Cd concentration was 3.6 mg and stem Cd concentration 5.6 mg kg^{-1} DW, and mean yield of seeds 2 tonnes ha^{-1} and stem 4 tonnes ha^{-1}, the total Cd uptake from 1 ha per vegetation season is ca. 30 g ha^{-1}. Thus, time needed for complete Cd decontamination is 9,000 g^{-1}/30 g = 300 years. Similar phytoextraction potential we have estimated on tenfold higher soil Cd concentration (30–35 mg Cd kg^{-1} soil; Bjelková et al. 2011a, b). Kos et al. (2003) estimated flax phytoextraction potential of above-ground tissues as 49 g Cd ha^{-1}, 1.99 kg Pb ha^{-1} and 0.70 kg Zn ha^{-1}; phytoextraction potential for hemp was calculated as follows: 9.57 kg Pb ha^{-1}, 3.68 kg Zn ha^{-1}, and 44 g Cd kg ha^{-1} (EDTA-stimulated improved HMs uptake from soil); unfortunately, developmental stage of flax and hemp plant at the harvest was not provided by authors and calculation was partly done based only on literature data on above-ground dry matter biomass. It was reported earlier that maximum concentrations of HMs were determined in vegetative growth and the final concentrations in the plant maturity are substantially lower (Cieslinski et al. 1996; Linger et al. 2005). All these facts are necessary to be taken into account during the estimation of phytoextraction potential—only mature plants have a sense in that relation. Linger et al. (2002) estimated hemp phytoremediation potential as 126 g Cd ha^{-1} per vegetation period. The same authors increased in the their next study this estimation up to 830 g Cd ha^{-1} per vegetation period (on soil with 17.3 mg Cd) (Linger et al. 2005). Based on these observations/estimations, we can expect that Cd-phytoextraction potential of flax/linseed on medium-polluted soils will not overcome 50 g Cd ha^{-1} per season, in case of hemp this value may be 10 times higher. In such a case, the complete decontamination of soil from Cd may be hypothetically shortened on some tens of years. Nevertheless, in reality the problem is not so simple due to need for crop rotation (in order to avoid soil-borne diseases attack as a result of repeated cultivation on the same field). Examples of HMs absorption by some fibre crops are provided in Table 11.6.

Table 11.6 Examples of HMs absorbtion from square unit (g ha^{-1}) by fibre crops above-ground biomass[a]

Plant species	HM phytoextraction	Reference
Cotton (*Gossypium hirsutum*)	Cu: 28; Fe: 626; Mn: 388; Zn: 103	Mullins and Burmester (1993)
Fibre flax (*Linum usitatissimum*)	Pb: 39; Cu: 54	Grzebisz et al. (1997a, b)
	Cd: 49; Pb: 1,990; Zn: 700	Kos et al. (2003)
Sida (*Sida hermafrodita*)	Cu: 30.6; Cd: 25.8; Pb: 27.1; Ni: 32.2; Zn: 387.2; Co: 10.8; Mn: 156.9; Fe: 430.8; Cr: 6.2	Borkowska et al. (2001)
Hemp (*Cannabis sativa*)	Pb: 141; Cu: 377	Grzebisz et al. (1997a, b)
	Cd: 44; Pb: 9,570; Zn: 3,680	Kos et al. (2003)
	Cd: 126–830	Linger et al. (2002, 2005)

[a]No calculation of time needed for total/partial soil decontamination was reported in above mentioned papers (speculative estimation = hundreds to tens of years)

11.6 Management and Industrial Processing of Contaminated Biomass

One of the crucial components of phytoextraction technology is the management and potential industrial processing of HMs-contaminated biomass. As compared to other agricultural crops producing sufficiently great biomass and accumulating heavy metals, flax and hemp has indisputable advantage in the extensive (multipurpose) and complete industrial utilisation of harvested biomass (Brouwer 2000; Karus and Vogt 2004). The use of flax and hemp in textile industry (natural fibres; supplement to synthetic fibres—linen fabrics, geotextile, agrotextile, insulation and filtration), pulp-and-paper industry, building and furniture industry (reinforced particle boards; reinforced building materials—paintings, concrete; composite polymers; insulation materials), chemical industry (oils, paints and varnishes), car and airplane industry (inside-door panellings) as well as energy crop (bales of straw, combustible briquettes, biopetroleum) was in last decade many times documented (Domier 1996; Murphy et al. 1997; Baraniecki et al. 1995; Štaud and Bjelková 1997; Brouwer 2000; Karus and Vogt 2004). After decades of high-tech developments of artificial fibres like carbon, aramid and glass it is remarkable that natural grown fibres have gained a renewed interest, especially as a glass fibre substitute in automotive industries. Fibres like flax, hemp or jute are cheap, have better stiffness per unit weight and have a lower impact on the environment (Brouwer 2000). Only increased content of heavy metals in fibre processed for clothing would represent some healthy risk and should be carefully monitored (Lukipudis, 2001; Öko Tex Standard 2005; Szynkowska et al. 2009; Table 11.7). Other industrial products practically do not represent any healthy risk. Also linseed oil is during seed processing (pressing) get off the heavy metals (Wislicki et al. 1997; Hocking and McLaughlin 2000). Thus, heavy metal contaminated flax and hemp raw material may be processed for many kinds of industrial products with added value which fact significantly decreases the cost of potential phytoremediation.

Table 11.7 OEKO-TEX® Limit values and fastness

Product Class	I Baby	II In direct contact with skin	III With no direct contact with skin	IV Decoration material
Extractable heavy-metals (mg kg^{-1})				
Sb (Antimony)	30.0	30.0	30.0	
As (Arsenic)	0.2	1.0	1.0	1.0
Pb (Lead)	0.2	1.0[a]	1.0[a]	1.0[a]
Cd (Cadmium)	0.1	0.1	0.1	0.1
Cr (Chromium)	1.0	2.0	2.0	2.0[b]
Cr (VI)	Under detection limit[c]			
Co (Cobalt)	1.0	4.0	4.0	4.0
Cu (Copper)	25.0[d]	50.0[d]	50.0[d]	50.0[d]
Ni (Nickel)[e]	1.0	4.0	4.0	4.0
Hg (Mercury)	0.02	0.02	0.02	0.02
Heavy metals in digested sample (mg kg^{-1})[f]				
Pb (Lead)	90.0	90.0[a]	90.0[a]	90.0[a]
Cd (Cadmium)	50.0	100.0[a]	100.0[a]	100.0[a]

[a]No requirement for accessories made from glass
[b]For leather articles 10.0 mg kg^{-1}
[c]Quantification limits: for Cr(VI) 0.5 mg kg^{-1}, for arylamines 20 mg kg^{-1}, for dyestuffs 50 mg kg^{-1}
[d]No requirement for accessories made from inorganic materials
[e]Including the requirement by EC-Directive 94/27/EC
[f]Applicable to all non textile accessories and components as well as for spun dyed fibres and articles containing pigments

In fact, there is a limited knowledge on distribution of accumulated HMs in specific tissues/cells of industrial (technological) importance (fibre cells or filaments, fibre bundles). There are several studies dealing with HMs contamination of flax and hemp fibre (the main raw material of fibre crops). Linger et al. (2002) concluded the fact that hemp accumulates heavy metals (provided data for Cd, Pb and Ni content in fibres and hurds) limits its use as a raw material in clothes. However, the high quality of the fibres and hurds, which were not affected by the HMs contamination, allows them to be used in special products like composite materials, where the fibres are embedded in polymers and could not be set free. Fibre bundle fineness and strength were not influenced by HMs contamination. Based on these facts authors considered hemp ideal candidate as profit yielding crop when used for phytoremediation purposes. Löser et al. (2002) were more critical to use HMs contaminated raw material, nevertheless, they used in their study heavily contaminated river sediment which resulted in relatively high accumulation of Cd in fibres.

There are several industrial processing variants of HMs-contaminated biomass:

- Fibre and whole biomass contains excessive amounts of HMs: Energy use (combustion, recyclation of ash HMs).

- Fibre contains HMs over accepted limits for garment textiles (Öko Tex Standard 2005; Table 11.7): composite materials, paper industry, geotextile and other industrial applications.
- Fibre contains acceptable amounts of HMs (Öko Tex Standard 2005; Table 11.7) or HMs are mainly concentrated in tissues out of fibre: garment textile industry, shives either burnt or used for other industrial applications.

This strategy may be changed as new scientific knowledge emerges or new plant materials will be produced (e.g. transgenic fibre crops with novel traits as related to HMs—Vrbová et al. 2012).

11.7 Conclusion: Concept of the Use of Flax and Hemp for Phytoextraction of Heavy Metals from Polluted Agricultural Soils

Nearly 25 years of research of two important fibre crops—flax and hemp—enable to make some conclusions and to formulate a strategy for future research and exploitation of HMs phytoextraction technology. The critical assessment of literature sources and our own long-term experience with both crops helped to make a more or less realistic view on the problem.

Unfortunately, the expectations were not fulfilled that hyperaccumulators will be found within commercial cvs and germplasm resources of the both crops—in flax/linseed, several hundreds of genotypes were tested for a trait "HMs tolerance and accumulation", nevertheless, the genotypes with desirable level of accumulation of Cd, Pb and other toxic elements in above-ground biomass have not been recorded. The limited number of up-to-date studied genotypes in hemp still offers a chance to find the hemp genotypes of interest. First successful results of genetic engineering of flax for HMs tolerance and accumulation (Vrbová et al. 2012) represent a promising approach how to improve phytoremediation potential of fibre crops.

On the other hand, the positive point of reported results is that medium or even high soil concentrations of metal elements do not have negative effect on the growth, yield of biomass and technological quality of raw material of both crops (here mainly fibre), and thus the production of flax and hemp from HMs polluted areas may be sold and industrially processed. The medium values of phytoextraction potential, i.e. tens or hundreds g. HMs ha^{-1} per vegetation period result in time needed for complete decontamination ca. in tens or hundreds years (Böhm et al. 1992; Linger et al. 2002). Based on these facts it is evident that flax and hemp cannot clean up the heavily metal-polluted sites in a short time, but they may have a specific role in successive decontamination of agricultural soils by their incorporation to specifically designed crop rotation systems on soils polluted by heavy metals (Grzebisz et al. 1997b). The idea is a gradual decreasing of heavy metal content to natural levels in the reasonable period of time in order to make possible the use of these soils for food production purposes again. The possibility of further industrial

processing makes the flax/linseed and hemp economically interesting crops for farmers/operators of phytoextraction technology (Griga et al. 2003a, b).

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Chapter 12
Transgenic Approaches to Enhance Phytoremediation of Heavy Metal-Polluted Soils

Pavel Kotrba

12.1 Introduction

Contamination of soils and sediments with toxic heavy metals contributes to serious environmental, economic, and health problems. Plants are predominant organisms in most ecosystems and have the natural ability to take up toxic metals along with micronutrients (Sarwar et al. 2010; Kabata-Pendias 2011). A promising and relatively new technology, referred to as phytoremediation, offers benefits of affordable and environmentally sustainable in situ bioremediation method (Pilon-Smits 2005; Macek et al. 2008; Doty 2008; Kotrba et al. 2009; Aken et al. 2010; Bhargava et al. 2012; Rajkumar et al. 2012). The phytoremediation approaches considered particularly suitable for reclamation of metal-polluted soils are phytoextraction and phytovolatilization. Phytoextraction aims at use of metal-accumulating plants that concentrate the pollutant in aboveground harvestable parts. Phytovolatilization is a process by which plants allow the accumulated pollutant to evaporate through their leaf surface when converted *in planta* to volatile forms. There are also other tactics relevant to phytoremediation of inorganic xenobiotics. In phytostabilization, plants are employed to prevent migration of contaminants to sites where they may pose a danger, and in rhizofiltration plant roots are used to absorb, concentrate, and/or precipitate pollutants from contaminated effluents.

Soils with abnormally high concentrations of some of the elements vary widely in their effects on different plant species. Some plants, including several metallophyte crops such as Indian mustard (*Brassica juncea*) or sunflower (*Helianthus annuus*),

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This chapter is dedicated to the memory of my colleague, Professor Martina Macková (1965–2012).

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have an inherent ability to accumulate high concentrations of metals in the aboveground biomass (Kabata-Pendias 2011). Of particular interest are species, referred to as hyperaccumulators, that are able to accumulate in their shoots more than two and up to four orders of magnitude higher concentrations of heavy metals than other adjacent plants (Brooks 1998; Reeves 2006; Verbruggen et al. 2009; Krämer 2010). The term hyperaccumulation was coined by Jaffre et al. (1976) who reported an extreme phenotype of *Sebertia acumunata*. This species produces latex containing up to 26 g Ni kg^{-1}, probably the most extreme metal concentration reported in plants to date. Currently, the accepted concentration criterions in shoot tissues of hyperaccumulators on a dry-weight basis are >0.1 wt% for most metals, except, for example, for zinc (>1 wt%), cadmium (>0.01 wt%), or gold (>0.0001 wt%) (Baker et al. 2000). About 500 plant species of 34 families (0.2 % of angiosperms) worldwide have been identified as hyperaccumulators of heavy metals (Co, Ni, Cu, Zn, Cd, and Pb), metalloids (As), and nonmetals (Se) as well. With few exceptions, among them Zn- and Cd-hyperaccumulating *Sedum alfredii* (Crassulaceae) or Co- and Cu-hyperaccumulating *Aeollanthus subacaulis* (Lamiaceae), the plant families most strongly represented are the *Brassicaceae* (in particular *Alyssum* spp., *Thlaspi*/*Noccaea* spp., and *Arabidopsis halleri*), *Euphorbiaceae*, *Asteraceae*, *Flacourtiaceae*, *Buxaceae*, *and Rubiaceae* (Reeves 2006). Regrettably, the use of hyperaccumulators for large-scale phytoextraction is severely limited because of their slow growth, low biomass, and often tight association with a specific habitat and lack of good agronomic characteristics (Cunningham et al. 1995; Chaney et al. 2005; Meyer and Verbruggen 2012).

For phytoremediation-based reclamation of metalliferous soils to be successful, plants should produce high biomass and accumulate and tolerate in their shoots high levels of toxic metal species (Pilon-Smits 2005; Bhargava et al. 2012). The focus for enhanced phytoremediation of soil metals is thus to use eligible plants with higher biomass and well-established agriculture. Common high-biomass crop plants or fast growing trees, such as poplar or willow, can be triggered to accumulate high amount of metals by enhancing the mobility of metal from the roots to the green parts of the plant by adding mobilizing agents when the crop had reached its maximum biomass (Huang and Cunningham 1996; Blaylock et al. 1997; Le Cooper et al. 1999; Chen et al. 2004; Chaney et al. 2007). Though this approach results in decontamination of soil it involves chemical intervention to the soil, thereby causing secondary pollution. A suggested step forward for making phytoremediation a viable technology is enhancing the metal accumulation and tolerance by overexpressing in transgenic plants the genes involved in homeostasis, metabolism, uptake, and translocation of the toxic elements. This chapter examines suitable targets, outcomes, and prospects of transgenic plant research towards upgraded phytoremediation plants.

12.2 Risks Associated with the Use of Transgenic Plants and Risk Mitigation Strategies

The impact that the introduction of transgenic phytoremediation plants into the outdoor environment would have on biodiversity and general safety should be carefully evaluated and weighted against known disadvantages of conventional remediation techniques or risks of having the recalcitrant heavy metal species in our environment. Since the introduction of the first genetically modified crops, a large number of safety-assessment studies have been devoted to the potential and imagined risks of transgenic plants for agricultural use (Singh et al. 2006; Kok et al. 2008; Kwit et al. 2011). Unlike with genetically modified crops, the issues such as food safety or allergenicity are not relevant when transgenic plants are considered for use in phytoremediation. Some risk assessment methods suggest that the danger of entry of metals to food chains through genetically modified accumulator would be low in most cases, because such plants would be in isolated industrial districts. However, an improved tolerance to toxic metals implemented through genetic engineering would provide modified plants with a selective advantage at contaminated sites, for example, with acquired metallotolerance (Linacre et al. 2003; Davison 2005). Potential changes in biological diversity due to invasion of privileged transgenic plants and the effects of they may exert on related soil microorganisms, herbivores and other organisms along the food chain must be also taken into account.

The main risk concerns the gene flow from cultivated plants to wild relatives via cross-pollination. The threat of uncontrolled pollination and crossing with the relatives and spreading of seeds can obviously be often avoided by harvest before flowering. In addition, various genetic methods are available that would restrict transgenic flow in a self-maintaining manner and facilitate the use of transgenic plants also in remediation of contaminated countryside farmlands. Most of these strategies were developed to target pre-hybridization steps; far fewer target post-hybridization events. One approach is targeting the heterologous gene into chloroplasts. Chloroplast DNA is maternally inherited and its transmission via pollen occurs rarely, though a danger of transfer of a functional heterologous gene nuclear DNA and thus to the nucleus of the next generation pollen exists (Kwit et al. 2011). A suitable technique restricting the spread of transgenes by seeds is based on poison/antidote idea and employs lethal ribonuclease barnase of *Bacillus amyloliquefaciens* as poison and protein barstar as antidote (Kuvshinov et al. 2001). To implement poison/antidote pathway, the plant is also transformed with the barnase and barstar genes. The barnase gene is controlled by the promoter, which is only active at the time of seed-pod development. Expression of barstar gene is regulated by heat-shock promoter. Correct seed development and germination is possible only when the barstar is produced due to the controlled heating of developing seeds to 40 °C. Such conditions are unlikely in the field, making the germination of progeny likely to fail there. A strategy highly appropriate in phytoremediation crops, whose primary purpose is not tied to fruit or seed

development, is disruption of pollen fertility. For example, inactivation of the UDP-glucose pyrophosphorylase 1 gene for flower development has resulted in a male sterile phenotype in rice (Woo et al. 2008). Post-zygotic methods may involve transgene placement in the crop loci that confer lower fitness and competitiveness to the wild relative and are thus negatively selected in the wild plants (Kwit et al. 2011). It is difficult to achieve with random T-DNA-based transformation, but transgene insertion at a targeted locus is currently feasible using zinc-finger nuclease technology (Li et al. 2009; Shukla et al. 2009). The creation of selectively terminable transgenic lines represents another strategy, as demonstrated in rice by the tagging of a gene of interest with an RNAi cassette that suppresses the bentazon detoxification gene *CYP81A6* (Lin et al. 2008). This has resulted in the creation of rice that is sensitive to a major herbicide, bentazon. Therefore, any possible hybrids outside of the field could be controlled by spraying bentazon during the conventional rice weed control process.

Use of antibiotic or herbicide-resistance genes as a simple method to select for a transformation event is often criticized, although the risk of their horizontal transfer from engineered plant is essentially negligible (Bennett et al. 2004). The more realistic threat is, however, the mobilization of genes and elements proximal to the gene for antibiotic resistance, which is always also the heterologous gene-of-interest. As genetic determinants of antibiotic resistance are widely distributed in the environment, a potential mechanism of horizontal transfer involving homologous recombination exists. The avoidance of antibiotic or herbicide markers is thus encouraged. For example, the plants harboring antibiotic resistance transgenes are no longer authorized for application in the EU since 2005 (EU directive 2001/1//EC). In this context, alternative selection systems are being developed, including positive selection employing the *E. coli pmi* encoding a phosphomannose isomerase (Bojsen et al. 1998). Since mannose-6-phosphate formed from mannose *in planta* is toxic to glycolysis in plants and Pmi enzyme converts this compound to natural metabolite fructose-6-phosphate, *pmi*/mannose system offers benefits of positive selection. However, the best solution to the selection marker problem is the precise excision of the marker gene from a chromosome using site-specific recombinases, such as Cre, FLP, PaA, or PhiC31 (Zuo et al. 2002; Gilbertson 2003; Thomson et al. 2009; Kempe et al. 2010). This strategy would then render transgenes containing only those heterologous genes, which are to be employed for the phytoremediation job.

12.3 Molecular Targets to Genetic Manipulation in Plants

Prerequisite to the accumulation of metal in the aboveground tissues is its mobilization from soil, metal uptake and root-to-shoot translocation mechanism, and competence to detoxify (over)accumulated metal species (Clemens et al. 2002). Tight control and regulation of accumulation and homeostasis thus evolved in all plants for essential metals and is of central importance both at organism and cellular level. Uptake of nonessential metals employs the same mechanisms as adopted by

essential metals (Krämer et al. 2007). Unless detoxified, nonessential metal ions may exert their toxic effect at virtually any tissue and cellular concentration. The property of hyperaccumulators to concentrate in their tissues heavy metal ions in large quantities is probably a consequence of their adaptation (Verbruggen et al. 2009). However, the selective factors causing the evolution of hyperaccumulation, which required complex alterations in the plant metal homeostasis network, are unknown and difficult to identify retrospectively. It has been suggested that accumulated metals execute defense function, poisoning plant tissues for herbivores and pathogens (Boyd 2007; Noret et al. 2007).

12.3.1 Heavy Metal Uptake and Translocation

The actual bioavailability of metal ions in soil is limited, because of their presence in mineral form, formation of hydrous oxides at pH >5, and strong binding to soil components like humic and fulvic acids. The soil microflora can modulate the bioavailability of metals by several mechanisms (Gadd 2007, 2010). Metabolic activities of some microorganisms may result in immobilization of metallic species in soil by such mechanisms as organic precipitation with oxalates, inorganic precipitation with carbonates, phosphates, or hydroxides, redox immobilization, sorption at cell walls and associated polymeric substances, and bioaccumulation. Some microorganism may, in turn, mobilize metals through excretion of H^+ and carboxylic (e.g., citrate) ligands and redox conversion to mobile forms. Also plants can solubilize metals for uptake by decreasing pH within the rhizosphere or by various organic chelators (root exudates; Fig. 12.1), such as carboxylates or phytosiderophores from the mugineic acid family (Chaney et al. 2007; Nair et al. 2007). Although the concept of developing transgenic plants with enhanced secretion of such ligands is plausible, there is no definite answer to the question of whether, and how, would such modification promote the metal uptake.

Following mobilization, the initial contact of the metal ion with root cell involves its biosorption at the cell wall via ion-exchange and chelation at cellulose, hemicellulose, pectin, and some minor polymers. The transport of most divalent heavy metals into root cells (Fig. 12.1) seems to be driven by members of the zinc-regulated transporter, iron-regulated transporter (ZIP) family (Krämer et al. 2007; Migeon et al. 2010). It is of particular interest to note that, unlike non-hyperaccumulating species, hyperaccumulator *Noccaea caerulescens* (alpine pennygrass; previously named *Thlaspi caerulescens*) constitutively overexpress in its roots *ZIP1* gene, whose products mediate high-affinity Zn transport as well as low-affinity Cd uptake (Pence et al. 2000; Hammond et al. 2006; van de Mortel et al. 2006; Milner et al. 2012). Gene expression analyses in *N. caerulescens* and in another Zn-, Cd-hyperaccumulator *A. halleri* have further highlighted overexpression of more ZIP members and physiological studies provided strong evidence that multiple uptake system are involved in the root uptake of Cd and Zn, which show differential preference for these metal ions (Lin et al. 2009; Verbruggen

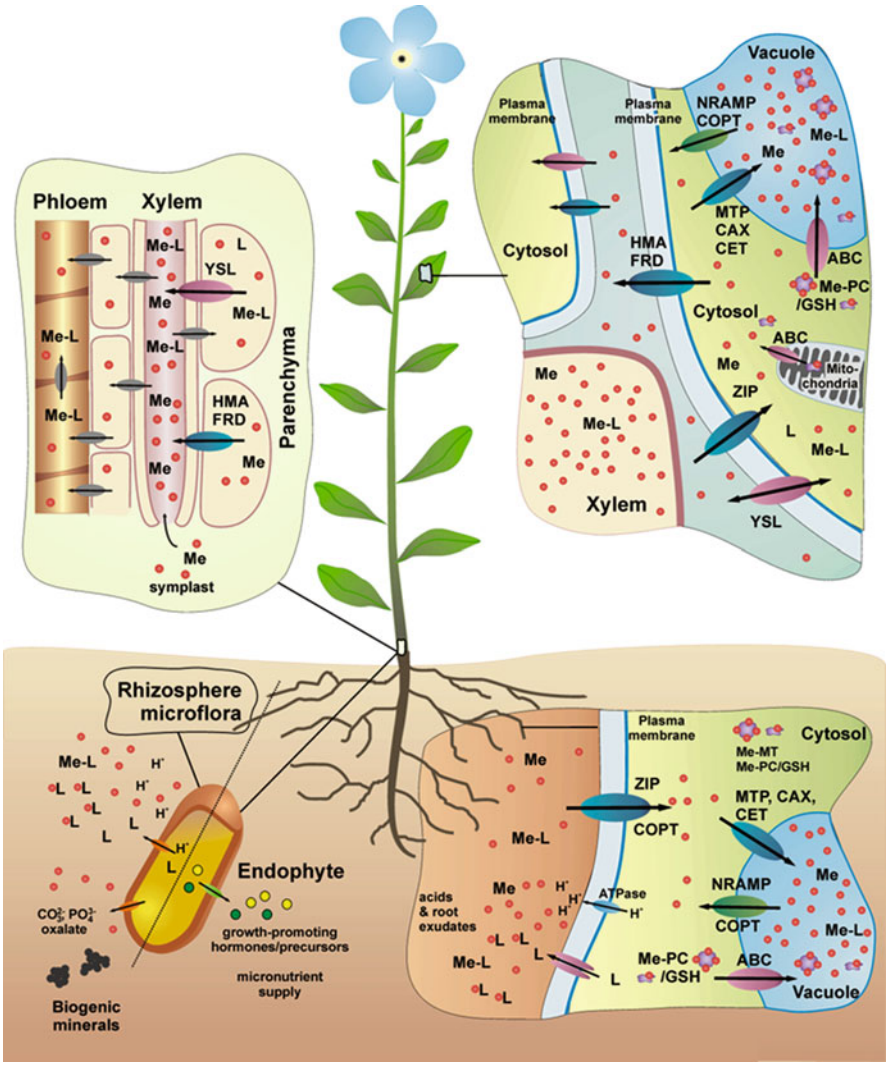

Fig. 12.1 Molecular determinants and mechanisms involved in metal–plant interaction. The metal is mobilized in the rhizosphere by secreted acidic or chelating molecules of both plant (root exudates) and microbial origin. The soil microflora may also trigger formation of organic or inorganic secondary minerals (carbonates, phosphates and hydroxides increases, oxalates), rendering the metal less available to plant. Endophytic bacteria inhabiting the plant mainly contribute mainly by promoting plant growth (support, e.g., the acquisition of micronutrients or the production of plant hormones). Root-to-shoot translocation of metals, either as hydrated ions or metal–ligand complexes, occurs via the xylem. Metals reaching the aboveground apoplast are than differentially captured in different cell types, moving also cell-to-cell through plasmodesmata (not shown). Transporters and transporter families involved in uptake and redistribution of metals within plant body and cells are indicated: *ZIP* zinc-regulated, iron-regulated transporter protein family; *COPT* copper transporter family (*syn.* CTR); *HMA* heavy metal ATPase of P_{1B}-type

et al. 2009; Krämer 2010). Although ZIP transporters, such as ZIP2 of *Arabidopsis thaliana*, may be involved also in import of Cu (Yamasaki et al. 2009), the high affinity uptake of this is being attributed to proteins of the Cu-transporter protein (COPT) family (Puig et al. 2007; Burkhead et al. 2009). Members of COPT (*syn.* CTR) family are well characterized from many eukaryotes as essential for Cu uptake and homeostasis (Puig and Thiele 2002). Moreover, the human plasma membrane hCTR1 has been demonstrated recently to execute also the high-affinity Ag^+ uptake (Bertinato et al. 2010). In *A. thaliana* COPT1 was the first to be identified as the root Cu uptake transporter essential for healthy plant growth (Sancenon et al. 2004). Moreover, overexpression *COPT1* or of the otherwise uncharacterized *COPT3* genes resulted in overaccumulation of Cu and sensitivity to Cu excess (Andres-Colas et al. 2010).

Efficient translocation of metal ions to aboveground organs requires radial passage from root symplast to xylem apoplast (Fig. 12.1). Here we refer to the symplast–apoplast concept, which considers that all the cells of higher plants are connected forming symplast. Continuous semipermeable membrane then separates the symplast from the apoplast, the nonliving parts of the plant tissue (cell walls, xylem, and intercellular space). The passage of metal ions from root cells into apoplast (xylem) occurs via specific membrane transporters and is generally tightly regulated. Among them, P_{1B}-type ATPases, in plants referred to as HMA (heavy metal ATPase) transporter, were characterized from both non-hyperaccumulators and hyperaccumulating species. Transporters of this type play an important role in transporting heavy metal ions against their gradient in virtually all kingdoms of life (Argüello et al. 2007; Migeon et al. 2010) on the expense of ATP (hence the name ATPases). They constitute two functional groups: those transporting monovalent metal ions (Ag, Cu) and those transporting divalent metal ions (Zn, Cu, Co, Cd, Pb). In bacteria and certain fungi, P_{1B}-ATPases are among the main players in the metal tolerance (Silver and Phung 2005; von Rozycki and Nies 2009). Since the characterization of the AtHMA4 of the divalent group from *A. thaliana* (Mills et al. 2003, 2005), the role of its homologues in hyperaccumulating species is being well established. The key difference that seems to greatly contribute to hyperaccumulation is triplication and quadruplication of *HMA4* genes in genomes of *A. halleri* and *N. caerulescens*, respectively (Hanikenne et al. 2008; Ó Lochlainn et al. 2011). In hyperaccumulating *A. halleri* as well as in *N. caerulescens*, HMA4 gene is more expressed in both roots and shoots compared with their non-hyperaccumulating Zn- and Cd-sensitive relatives (Verbruggen et al. 2009),

Fig. 12.1 (continued) ATPase family; *YSL* yellow-stripe 1-like transporters of OPT (oligopeptide transporter) family; *FRD* Fe-citrate transporter "ferric reductase defective"; *ABC* ATP-binding cassette family; *MTP* metal transporter proteins of CDF (cation diffusion facilitator) family (MTP in *B. juncea* also referred to as cation efflux transporters [CAT]); *CAX* Ca/cation exchanger family; *NRAMP* natural resistance-associated macrophage protein family. *Me* metal; *L* general ligand (e.g. citric acid, nicotianamine; extracellular [phyto]siderophores); *MT* metallothionein; *GSH* glutathione; *PC* phytochelatin

consistent with the idea that HMA4 is responsible for root detoxification by translocating the metal ions to aboveground tissues. Indeed, RNAi-mediated silencing of *HMA4* gene in *A. halleri* rendered plants which accumulated less Cd and Zn in shoots and were more metal sensitive (Hanikenne et al. 2008). A member of the monovalent group of P_{1B}-ATPases from *A. thaliana*, HMA5, has been reported to be crucial for vascular translocation of Cu (Andres-Colas et al. 2006; Burkhead et al. 2009). Several lines of evidence also suggest that efficient Cu transport by HMA5 requires cytosolic ATX1 copper-binding chaperone, to which a role in funneling of the metal ion to the membrane transporter is being attributed (Puig and Thiele 2002; Shin et al. 2012). Other important transport proteins implicated in the heavy metal translocation are yellow-stripe 1-like (YSL) transporters of oligopeptide transporter (OPT) family; hence they transport metal chelates rather than free-hydrated cations. Members of this family are abundant in *A. thaliana* and other non-hyperaccumulating species where they respond to Fe availability (Krämer et al. 2007; Migeon et al. 2010). There is striking evidence for a role of at least two YSL transporters in the Zn and Ni hyperaccumulation of *N. caerulescens*, in which YSL3 was shown to transport Ni-nicotianamine complexes (Gendre et al. 2007; Haydon and Cobbett 2007). In plants, metal chelates (Fe-citrate) are also transported by FRD3 transporter of multidrug-resistance transporter family and high expression levels of *FRD3* genes in *A. halleri* and *N. caerulescens* compared to those in *A. thaliana* may suggest a role of FRD3 also in Zn translocation (van de Mortel et al. 2006; Talke et al. 2006).

The metal ions translocated from roots are in shoots subjected to redistribution through both apoplast and symplast. This is achieved by transporters of the same families, which are involved in the metal uptake and its radial passage. Thus xylem unloading or uptake from general apoplast to symplast is by ZIP transporters and the distribution to intercellular apoplast is by HMA/FRD/YSL transporters (Krämer et al. 2007; Migeon et al. 2010). Several lines of evidence suggest that for xylem transport free-hydrated metal ions are used, rather than complexes (Salt et al. 1999; Lu et al. 2008; Ueno et al. 2008). The possible translocation of metal complexes with peptide ligands (e.g., phytochelatins; see Sect. 12.4.2) via tissue symplast, initiated by their export via transporters of ATP-binding cassette (ABC) family, has been suggested by Bovet et al. (2005). High phytochelatin content and four times higher Cd levels in the phloem sap, compared to xylem, in the metallophyte rapeseed *Brassica napus* (Mendoza-Cózatl et al. 2008) provide some support to this idea. The role of ABC transporters is well established in vacuolar sequestration of the metal complexes (see Sect. 12.4.2), but their role in the metal-chelate translocation remains to be elucidated.

12.3.2 Intracellular Sequestration and Detoxification of Heavy Metals

A common feature underlying the interactions of heavy metal with the components of a biological system is relatively high reactivity of metal ions, mostly due to their ability to form coordination and covalent complexes. Upon the entry into the root

symplast, virtually all heavy metal ions are thus sequestered by specialized ligands or metallochaperones, which are together intimately involved in management of the storage metal pool. As many metallic species exert their toxic effect by induction of reactive oxygen species (ROS) and other free radicals, their elimination is another challenge faced by the cell (Foyer and Noctor 2005). Some heavy metal ions are essential for specific metabolic process, but may impair biological equilibrium when over accumulated. For the heavy metal (hyper)accumulation to be highly effective, the plant should be thus capable to maintain metal homeostasis and create appropriate metal sinks. The main detoxification mechanisms on subcellular level involve sequestration of the metal ion by ligands in cytoplasm eventually followed by transport of the metal (complex) into vacuoles. Other aspect relevant to (hyper) accumulation of heavy metals in aboveground tissues is their availability for translocation that also implies limited sequestration in or efficient mobilization from vacuoles of the root cells (Fig. 12.1).

The cysteine-rich metallothioneins (MTs) are intracellular ligands capable of tight coordination of heavy metal ions via cysteine residues shared along the peptide sequence in Cys–X–Cys or Cys–Cys motifs (X represents any amino acid). Peptides of MT family have been identified in plants, animals, eukaryotic microorganisms, and certain prokaryotes. Most of plant MTs consist of about 63–85 amino acids with two terminal cysteine-rich domains separated by a central region without any Cys residues and they cluster into four types (Freisinger 2008). Like animals and fungi, plants have several MT genes and MTs play a major role in the homeostasis of essential heavy metals and the transcription of their genes is controlled by signals instrumental during germination, organ development, and senescence (Clemens 2006). Animal and certain fungal MTs are, besides their function in homeostasis, known for their essential role in detoxification of toxic heavy metal ions (Coyle et al. 2002; Bellion et al. 2006; Vašák and Meloni 2011). In plants, MTs seem to be contribute to Cu tolerance in various Arabidopsis ecotypes and in *Silene vulgaris* (Murphy and Taiz 1995; Jack et al. 2007) and may be intricately involved in phloem Cu transport in *A. thaliana* (Guo et al. 2008b). In *N. caerulescens* the role of MTs is also attributed to Cu homeostasis and expression of type 3 MT was found particularly strong in Cd-hyperaccumulating populations (Roosens et al. 2004, 2005; Hassinen et al. 2007).

Intracellular detoxification of most heavy metal ions in plants and in certain yeasts relies on phytochelatins (PCs). These peptides of general structure $(\gamma\text{-Glu–Cys})_n$X (PCn; $n = 2$–11; X represents Gly, Ser, β-Ala, Glu, Gln, or no residue) tightly sequester multiple metal ions in metal–thiolate complexes, rendering them inactive in cellular processes (Clemens 2006). The metal–PC complexes formed in cytosol could further be deposited in vacuoles, which serve as cellular sink for toxic metal species (Fig. 12.1). In the yeast *Schizosaccharomyces pombe*, it involves the transport of the complex via ABC transporter Hmt1 (Clemens and Simm 2003) and its functional homologue Abc2 from *A. thaliana* has been characterized recently (Mendoza-Cózatl et al. 2010). Inside the vacuole, the metal–PC can accommodate inorganic sulfide and CdS crystallites are formed (Clemens and Simm 2003; Clemens 2006). Alternatively, the complex dissociates and released metal cations

form metal chelates with organic acids such as malate or citrate. The same apparently holds true for the role these organic acids play in hyperaccumulators (Verbruggen et al. 2009).While PC synthesis is ubiquitous response of plants to heavy metal, especially Cd, exposure, their accumulation was only been found in Cd-sensitive populations of *N. caerulescens* and in non-accumulating ecotypes of *S. alfredii* (Schat et al. 2002; Sun et al. 2007). The biosynthesis of PCs via transpeptidation reaction from glutathione (γ-glutamylcysteinylglycine, GSH) or its homologues (*iso*-PCs) is catalyzed by the constitutive PC synthase (PCS) in a metal- or metalloid (e.g. As)-dependent manner (Clemens 2006).

Glutathione (GSH) and its homologues also act as a fundamental antioxidant molecule. Glutathione directly eliminates reactive oxygen radicals induced by heavy metals in cells (Schützendübel and Polle 2002) and provide reducing equivalents in the ascorbate–glutathione antioxidation cycle to maintain redox homeostasis (Foyer and Noctor 2005). Such function is also attributed to GSH in Ni- and Cd-hyperaccumulating populations of *N. caerulescens* (Freeman et al. 2005; van de Mortel et al. 2008). In yeast, *Saccharomyces cerevisiae* (Li et al. 1997), and in some ectomycorrhizal fungi (Bellion et al. 2006) which do not produce PCs, cellular detoxification of Cd depends upon exclusion of bis (glutathionato)cadmium complex the metal into vacuoles. The differential Cd stress-dependent expression of homologues of the respective vacuolar ABC transporter (YCF1 in *S. cerevisiae*) has been reported in *A. thaliana* (Bovet et al. 2005). In this plant, GSH also appears to play a role in Cd sequestration in the mitochondria and bis(glutathionato)cadmium conjugates, transported via ABC transporter ATM3 into cytoplasm, become substrates for PC synthesis (Kim et al. 2006). Two homologues of *A. thaliana* MRP10 and ATH13 ABC transporters were found differentially expressed in *N. caerulescens* populations displaying contrasting Zn tolerance (Hassinen et al. 2007); however, a direct evidence for their role in vacuolar sequestration of Zn is missing. Like ABC transporters, Ca/cation exchangers (CAX) seem to be involved in vacuolar sequestration of heavy metals, although they seem to transport free ion rather that a metal chelate. They are known to respond to heavy metal exposure both non-accumulators and hyperaccumulators (van de Mortel et al. 2006, 2008; Weber et al. 2006), in which their metal specificity remains to be elucidated.

Transporters of the cation diffusion facilitator (CDF) family play an important role in heavy metal homeostasis and detoxification (Krämer et al. 2007; Montanini et al. 2007; Migeon et al. 2010). In plants, these are called metal transporter proteins (MTPs) and mediate not only the transport of variety of metals (Zn, Fe, Cd, Co, Mn) to vacuoles but also to other organelles and to the apoplast. The best characterized *A. thaliana* CDF transporter MTP1 and its homologues from *A. halleri* (Arrivault et al. 2006) or poplar (Migeon et al. 2010) are apparently Zn-specific transporters responsible from Zn efflux from cytoplasm to vacuoles. High expression of *MTP1* gene observed in both roots and shoots of *A. halleri* thus seem to be involved in Zn hypertolerance trait of this species. Unlike MTP1 of *A. halleri*, MTP1 from metal accumulator *Thlaspi goesingense* MTP1 been reported to have a much broader substrate range, which was verified to mediate tolerance to Ni, Cd, Co, and Zn in

yeast cells (Persans et al. 2001). Later study suggested that MTP1 of *T. goesingense* localizes in plasma membrane (Kim et al. 2004), where it could mediate transport of metals to apoplast. In *B. juncea*, the CDF transporters functionally related to MTP1 of *T. goesingense* are CET2, CET3, and CET4 (Xu et al. 2009; Lang et al. 2011). They can transport Zn, Cd, and Co in yeast model, and are *in planta* upregulated under Zn and Cd stress. When overexpressed, they enhance tolerance to and accumulation in shoots of Zn and Cd.

Since heavy metal ions are often subjects to vacuolar sequestration immediately upon their entry to root cells, there is a need for their remobilization before translocation to aboveground tissues. This can be accomplished by vacuolar ZIP transporters or transporter of the natural resistance-associated macrophage protein (NRAMP) family. The primary biological function of *A. thaliana* NRAMPs appears to be in Fe homeostasis (Krämer et al. 2007). In *A. halleri* or *N. caerulescens* NRAMPs show differential tissue-specific expression, thereby suggesting they may be involved in hyperaccumulation trait of these species (Weber et al. 2004; Hammond et al. 2006; Talke et al. 2006; van de Mortel et al. 2006). A specific phenomenon associated with hyperaccumulation is that substantial portion of heavy metal ions in root cells escape vacuolar sequestration. This is because metal form in the cytoplasm complexes with nicotianamine (NA) or histidine (His). Synthesis of NA from three *S*-adenosyl-methionine (SAM) molecules is present in all plants and the role of NA seems to be in the movement of micronutrients such as Zn, Cu, or Fe throughout the plant (Schuler and Bauer 2011). In hyperaccumulators, both NA and His are elevated and strongly inhibit the retention of metals, particularly Ni, in vacuoles, rendering the complex ready to mobilization into the aboveground tissues (Ingle et al. 2005; Callahan et al. 2006, 2007; Mari et al. 2006; Richau et al. 2009).

12.4 Genetically Engineered Plants

The above paragraphs define the targets for genetic modifications of plants directed towards the improved phytoextraction of metals from soils and sediments. These lay in such pathways as (1) mobilization and uptake of metal from the soil, (2) competence of metal translocation to shoots via symplast or xylem (apoplast), including efficiency of xylem loading, (3) distribution to aboveground organs and tissues, (4) sequestration within tissue cells, (5) expulsion of accumulated metal to less metabolically active cells. Removal of Hg from contaminated soil by phytovolatilization could be achieved on implementation of enzyme activities promoting plants, (6) capacity to convert metals to volatile species for phytovolatilization. Research papers describing successful genetic engineering of high biomass (crop) species particularly suitable for phytoextraction of metals are listed in Table 12.1. Substantial body of information was also obtained with transgenic model species *A. thaliana* and *Nicotiana tabacum*. Although deposition of heavy metals in roots is not desirable in phytoextraction strategy, improved metallotolerance in such organ could be of importance during phytostabilization

Table 12.1 High biomass plants genetically modified for enhanced phytoextraction of heavy metals

Plant	Modification	Metal species affected	References
Indian mustard	Uptake and translocation	Cd^{2+}, Zn^{2+}	Xu et al. (2009), Bhuiyan et al. (2011a), Lang et al. (2011)
	Vacuolar sequestration	Cd^{2+}	Bhuiyan et al. (2011b)
	PC and GSH synthesis	Cd^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, CrO_4^{2-}, WO_4^{2-}, MoO_4^{2-}	Zhu et al. (1999a, b), Pilon-Smits et al. (2000), Reisinger et al. (2008)
	Sulfur metabolism	Cd^{2+}, VO_4^{3-}, CrO_4^{2-}, WO_4^{2-}, MoO_4^{2-}	Pilon-Smits et al. (1999), Wangeline et al. (2004), Lindblom et al. (2006)
Shrub tobacco	PC synthesis	Pb^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}	Gisbert et al. (2003), Martínez et al. (2006)
Aspen	PC synthesis	Pb^{2+}	Couselo et al. (2010)
Poplar	GSH synthesis	Cd^{2+}, Zn^{2+}	Arisi et al. (1997), Koprivova et al. (2002), Bittsánszky et al. (2005)
	Metallothionein	Cu^{2+}, Zn^{2+}	Balestrazzi et al. (2009), Turchi et al. (2012)
Flax	Metallothionein	Cd^{2+}	Vrbova et al. (2012)

of contaminated soils. Therefore some efforts, especially for improved phytostabilization and rhizofiltration, should also be directed to improve root sequestration by metal-complex formation and deposition in vacuoles.

12.4.1 Plants Engineered for Improved Metal Uptake and Translocation

The transport of essential metals or alkali cations across plasma membranes by means of primary and secondary active transporters is of central importance in the metal homeostasis network in all organisms. Relatively broad substrate specificity of transporters makes them a promising tool to improve toxic metal uptake for phytoremediation. The *N. tabacum* plasma membrane transporter NtCBP4 (calmodulin-binding protein), for example, is structurally similar to vertebrate and invertebrate K^+ and to nonselective cation channels (Arazi et al. 1999). Overexpression of entire *NtCBP4* resulted in transgenic tobacco in 20 % increased uptake and translocation of Pb to shoots, reflected in the higher sensitivity of

modified plants compared to wild-type (WT) controls (Arazi et al. 1999; Sunkar et al. 2000). Intriguingly, the same transgene conferred improved Ni tolerance, which was apparently due to NtCBP4-promoted Ni-exclusion by yet unidentified mechanism. Expression of the Zn-uptake transporter *NcTZN1* gene (ZIP family) from the ascomycete *Neurospora crassa* in *N. tabacum* substantially promoted accumulation of Zn, but not Cd (Dixit et al. 2010). When grown hydroponically in media amended with 70 μM Zn, the Zn concentrations in roots and shoots of transgenic tobacco were 6.5- and 2-fold higher than those in WT plants. Under Cd stress (200 μM Cd), *N. tabacum* expressing MTF-like transporter genes *BjCET3* and *BjCET4* from *B. juncea* showed higher Cd tolerance and tobacco producing CET4 accumulated twofold more Cd in shoots than control plant, while maintaining similar shoot biomass production with controls (Lang et al. 2011). The overexpression of hypothetical plant iron transporters of the NRAMP family in model plants *A. thaliana* or *N. tabacum* has been primarily conducted to assess their function in Fe homeostasis (Curie et al. 2000). It was also found that the overproduction of intrinsic AtNRAMP3 in *A. thaliana* markedly increased sensitivity of the transgene to Cd, consistent with an idea of remobilization of Cd from vacuole (Thomine et al. 2000). However, this phenotype was not accompanied by increase in the net Cd accumulation. Promoted metallotolerance and translocation of Cd and Pb was achieved in *B. juncea* upon expression of mitochondrial Cd-efflux ABC transporter *AtATM3* from *A. thaliana* (Bhuiyan et al. 2011a). The best performing transgenic lines contained in their shoots up to 2.5-fold higher levels of both metal ions than WT. It should be noted that AtATM3 is not expected to localize in plasma membrane in transgenic plant; still it can be intricately involved in translocation. The authors attributed enhanced translocation to increased Cd and Pb levels in the cytoplasm which stimulated PC and GSH synthesis as well as expression of several intrinsic metal transporters.

The feasibility of using bacterial metal transporters in plants was first demonstrated in *A. thaliana* transformed with *zntA* coding for Zn, Cd, and Pb P1-ATPase responsible for the metal-efflux-based metalloresistance of *Eschericha coli* (Lee et al. 2003). In transformed *A. thaliana*, localized ZntA on plasma membrane reduced the Cd accumulation in protoplasts by promoting release of preloaded Cd. Overall ZntA improved the tolerance of the ZntA plants and shoots of transgenic grown at these concentrations showed, respectively, decreased content of Cd and Pb, a desirable feature for crop plants to be safer from heavy metal contamination. In *N. tabacum*, the expression of a bacterial proton-motive force drive transporter of the NiCoT family from *Rhodopseudomonas palustris* increased the accumulation of Co in shoots of hydroponically (42 μM Co in media) grown twofold (Nair et al. 2012). On the whole plant level and compared to the control, transgenic plant accumulated up to 5 and 2 times higher concentrations of Co and Ni, respectively, while uptake of Fe, Cd, Zn, and Cu remained unaffected.

The widespread bacterial Hg resistance mechanism, based on the import of Hg into cytoplasm and its subsequent reduction to metallic mercury involves MerT and MerC as one of the plasma membrane transporters for the Hg uptake step (Silver and Phung 2005). In a model experiment with *merC*-expressing *A. thaliana*, the leaves when excised and submerged into a solution containing 100 μM Hg, showed more

than threefold increased rate of foliar Hg accumulation as compared to WT controls (Sasaki et al. 2006). However, MerC *Arabidopsis* seedlings also acquired a Hg hypersensitive phenotype. Overproduction of MerC would be still an attractive approach, seemingly viable when further supported with some form of Hg detoxification. Polyphosphate kinase *ppk* gene is in the bacterium *Klebsiella aerogenes*, a key enzyme in polyphosphate (polyP) synthesis. Nagata et al. (2006b) showed that expression of *ppk* in *N. tabacum* rendered plant more Hg tolerant, seemingly because of binding/precipitation of intracellular Hg ions by polyP. Transgenic *ppk* plants growing in soil with 10 nmol Hg g^{-1} were able to accumulate 6 times more Hg than WT plants (Nagata et al. 2006a). To promote further accumulation of Hg in *N. tabacum*, the *ppk* plant was transformed with a bacterial *merT* gene encoding Hg-uptake transporter MerT (Nagata et al. 2009). When the *merT*/*ppk* plants were grown hydroponically in the presence of 0.1–2.5 μM Hg, they had 1.3–3 times higher foliar concentrations of Hg than tobacco expressing mere *ppk*. To extend the use of this system to phytoextraction of organomercurial compounds, which could not be transported by MerT, the *merB*/*merT*/*ppk* tobacco was developed that expressed also *merB* gene (Nagata et al. 2010). This bacterial gene encodes organomercurial lyase used to liberate Hg from organomercurials (Silver and Phung 2005). The feasibility of this approach was demonstrated with *merB*/*merT*/*ppk* tobacco callus, which was more resistant to methyl mercury and accumulated more Hg from methyl mercury added to the culture media than *merT*/*ppk* or WT lines.

12.4.2 Plants Engineered for Improved Compartmentalization of Metals

Subcellular sequestration of metal ions may, besides chemical complexation by ligands in cytoplasm, involve the transport into vacuoles as the final metabolically inactive sink. Manipulation of vacuolar exchange activity in *N. tabacum* by the overproduction of the metal ion/H^+ antiporters CAX2 and CAX4 (calcium exchanger 2 and 4) of *A. thaliana* provided transgenic plants the ability to efficiently detoxify Cd, Zn, and Mn (Hirschi et al. 2000; Korenkov et al. 2007a, b). The CAX2 or CAX4 plants showed an improved uptake of metal ions in the roots but not in shoots, which accumulated 70–80 % less metals than the roots. However, the net metal uptake was elevated in shoots due to the acquired metal tolerance and markedly improved aboveground biomass yields (e.g., the Cd content of CAX2 and CAX4 plants grown in media with 3 μM Cd accumulated 3.4 and 2.4 times higher than that in WT). A site-directed mutagenesis approach was used to alter His338 of an activated N-terminal truncated form of *A. thaliana* CAX1 to obtain CAXcd variant with H338N substitution and high apparent Cd transport activity (Shigaki et al. 2005). When CAXcd was constitutively expressed in petunia (*Petunia* × *hybrida* Dreams™ Red), transgenic plants treated with either 50 or 100 μM Cd showed more vigorous growth compared with controls and accumulated up to 2.5-fold more Cd in their leaves than WT (Wu et al. 2011).

In yeast *S. cerevisiae,* Cd is detoxified by transport of cytosolic bis (glutathionato)cadmium complex to vacuoles by ABC-type YCF1 transporter (Li et al. 1997). Accordingly, heterologous expression of *YCF1* gene in *A. thaliana* rendered transgenic plant with an enhanced tolerance to Cd and Pb (Song et al. 2003). Quite surprisingly, the YCF1 plant also efficiently translocated these metals to shoots, which, compared to WT, accumulated 1.5 higher metal levels from media with 70 μM Cd or 750 μM Pb. Moreover, the same phenotype was observed in the *YCF1*-transformed *B. juncea* (Bhuiyan et al. 2011b). Transgenic lines showed better growth and 1.5- to 2.1-fold higher Cd and Pb content in shoots than did WT plants grown in the same hydroponic solutions containing 150 mM Cd of 1 M Pb. Although the expression of the mammalian *hMRP1* gene, encoding a different type the ABC-type multidrug resistance-associated transporter, did not alter Cd accumulation in the organs of *N. tabacum*, transgenes showed improved Cd tolerance compared to WT controls, manifested by the continuous growth of transgene plantlets, reduced chlorosis, and a 25 % faster root elongation on media containing 100–240 μM Cd (Yazaki et al. 2006). Mammalian ATP-binding cassette (ABC) transporters involved in the multidrug resistance of cancer cells can efflux cytotoxic compounds that show a wide variety of chemical structures and biological activities. Human multidrug resistance-associated protein (hMRP1) is one of the most intensively studied ABC transporters and many substrates have been identified, including both organic and inorganic compounds (Zhou et al. 2008). Interestingly, in mammals, members of the MRP family are found in plasma membrane, while in *N. tabacum*, hMRP1 is localized in vacuolar tonoplasts. Besides detoxification of Cd, presumably transported to vacuoles as glutathione–Cd conjugate, hMRP1 also conferred vacuolar uptake and resistance to model organic xenobiotic daunorubicin, an anthracycline-type DNA-intercalating drug, suggesting that MRP transporters could be beneficial in constructing plants for the remediation of a complex polluted environment (Yazaki et al. 2006).

In eukaryotic cells, intracellular membrane transport involves vesicle formation and fusion with a target membrane. This process is mediated by numerous components. In particular, the specificity of membrane fusion is mediated by membrane-associated proteins called SNAREs (soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptors) (Wickner 2010). An interesting approach to promote sequestration of Cd in vacuoles emerged from finding that *A. thaliana* SNARE proteins, SYP111 and SYP121 are involved in the transport of secretory vesicles at the plasma membrane, and AtVAM3 (SYP22) provides target SNARE function during the late stages of vacuolar assembly (Uemura et al. 2004). In the yeast model, these SNARE functions were capable to direct bacterial transporter MerC from the plasma membrane to the vacuolar tonoplast, thereby promoting vacuolar sequestration of Hg as well as Cd (Kiyono et al. 2010, 2011). More recently, Kiyono et al. (2012) demonstrated feasibility of this approach in *A. thaliana*. The transgenic seedlings with MerC-SYP121 fusion in the tonoplast were more resistant to Cd than WT and whole seedlings accumulated by 20 % more metal when grown hydroponically in the presence of 20 μM Cd.

12.4.3 Plants Engineered for Enhanced Metal Ligand Production

As explained in Sect. 12.3.2, PCs are synthesized by phytochelatin synthase (PCS) enzyme from glutathione or its homologues. Although the overexpression of intrinsic *AtPCS1* in *A. thaliana* resulted in 25 times higher levels of the transcript and up to a twofold increased production of PCs, *AtPCS1*-transformed lines paradoxically showed hypersensitivity to Cd and Zn (Lee et al. 2003). Such a phenotype could be attributed to a rapid nonphysiological decrease in the intracellular GSH pool due to the synthesis of supraoptimal levels of PCs. In contrast, expression of *TaPCS1* encoding PCS from wheat in shrub tobacco *N. glauca* substantially increased its tolerance of transgenic plants to Pb and Cd (Gisbert et al. 2003). Moreover, TaPCS1 *N. glauca* accumulated, respectively, 6, 3.3, 4.8, 18.2, and 2.6 times more Pb, Cd, Zn, Cu, and Ni from industrial soil than did the WT plant (Martínez et al. 2006). Also certain lines of transgenic aspen (*Populus tremula* × *tremuloides* cv. Etrepole) expressing the same *TaPCS1* gene showed better growth than the parental plant and accumulated more Pb from mining soil (Couselo et al. 2010). Transgenic plants also showed higher biomass and by 70 % higher Pb levels than WT in exposures to up to 1.5 mM Pb concentrations in the hydroponic growth media. Since GSH molecule is involved in many aspects of the plant response to heavy metal ions, many efforts have been directed towards engineering its biosynthesis pathway. Attempts to increase GSH production in plants, by the implementation of enzyme activities involved in its synthesis and recycling, have aimed mainly at the promotion of increased PC levels under metal stress. GSH is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), respectively. Constitutive production of the *E. coli gshI* gene and targeting of encoded γ-ECS in plastids in *B. juncea* increased GSH levels in hydroponically grown transformants threefolds (Zhu et al. 1999b). Consequently, the PC2 levels of shoots and PC2, PC3, and PC4 levels in roots of γ-ECS *B. juncea* stressed at 200 μM Cd increased, compared to WT plants, by 30 %, which resulted in higher Cd tolerance. In 50 μM Cd exposures, overexpression of *gshI* enhanced the natural capacity of *B. juncea* to accumulate Cd in shoots nearly twofold. The effect of cytosolic overexpression of *gshII* encoding GS on Cd tolerance and accumulation from a hydroponic solution was less pronounced, although transformed plants stressed at 100 μM Cd had 2.3 and 1.7 times higher PC2 compared to the WT control (Zhu et al. 1999a). Both *gshI* and *gshII* were later demonstrated to enhance the capacity of *B. juncea* to accumulate from hydroponic solutions and tolerate a variety of metals and metalloids (particularly As, Cd, and Cr) as well as mixed-metal(loid) combinations (Reisinger et al. 2008). Bennett et al. (2003) further demonstrated that overexpression of *gshI* and *gshII* can indeed promote phytoextraction with *B. juncea* in soils from a mine tailings: plants expressing *gshI* accumulated in shoots, respectively, 3.5, 2.0, 1.54, and 2.0 times higher levels of Pb, Zn, Cd, and Cu than the WT plants and those expressing *ghsII* contained in shoots 1.5 times higher concentrations of Cd and Zn than the

control WT. The same hold true for hybrid poplar (*Populus tremula* × *P. alba*), in which production of *E. coli* γ-ECS enhanced foliar GSH content 2- to 4-folds (Arisi et al. 1997). It promoted accumulation of Cd, but not of Zn, particularly in young leaves of plants grown in soils complemented with 225 mg kg^{-1} Cd; they contained 2.5–3.0 higher levels than WT (Koprivova et al. 2002; Bittsánszky et al. 2005). The significance of glutathione reductase (GR) in Cd accumulation and tolerance was recorded in transgenic *B. juncea* producing the GR of *E. coli* in the cytosol and plastids (Pilon-Smits et al. 2000). Only plastidic heterologous GR, improving natural enzyme levels 20- to 50-fold, doubled GSH levels in roots. In contrast to the WT control, the plastidic transformants showed no chlorosis when treated with 100 μM Cd; however, the shoot Cd accumulation was only a half of that of control WT plants.

As indicated above, the overproduction of PCs followed by an exhaustion of the GSH pool in *A. thaliana* had a negative impact on the ability of transgenes to tolerate and accumulate Cd (Lee et al. 2003; Li et al. 2004). This phenotype was converted to the tolerant on expression of yeast *GSH1*-encoded GS in *A. thaliana* lineages producing AsPCS1 of garlic *Allium sativum* (Guo et al. 2008a). Combination of both transgenes further enhanced by four times the natural capacity of *A. thaliana* to accumulate Cd from media with 30 mg kg^{-1} Cd. The sulfur assimilatory mechanism and subsequent production of the antioxidant and PC precursor GSH in plants is known to be highly induced by heavy metal exposure (Foyer and Noctor 2005). In the respective pathways, the overall rate of GSH biosynthesis and the capacity to maintain an elevated GSH pool limited by the activity of cysteine synthase (*O*-acetylserine [thiol] lyase, OAS-TL), which substitutes the acetate of *O*-acetyl-L-serine (OAS) with sulfide (Barroso et al. 1995; Meyer and Fricker 2002). Indeed, constitutive overexpression of *Atcys-3A* encoding intrinsic OAS-TL in *A. thaliana* increased intracellular cysteine and GSH levels, allowing transgenes to survive at 400 μM Cd stress (Domínguez-Solís et al. 2004). Over a 14-day period, OAS-TL Arabidopsis accumulated 72 % more metal than WT control plants from a medium containing 250 μM Cd, the highest Cd content being detected in the trichomes. Kawashima et al. (2004) reported a substantial improvement in Cd and Ni tolerance in *N. tabacum* overproducing OAS-TL from spinach (*Spinacia oleracea*). The authors also determined the Cd accumulation potential of the best performing transgenic line and found that the Cd concentration was reduced in roots (4 times) and slightly higher (1.4-fold) in shoots compared to the WT control, indicating the onset of promoted metal translocation. Moreover, due to highly improved biomass yields on media with 100 μM Cd, shoots of 3-weeks-old transgenic plants accumulated 2.8 times higher net amount of the metal than shoots of WT plants.

Improved supply of *O*-acetyl-L-serine (OAS) to the OAS-TL enzyme has also been shown as an effective method to increase the rate and yield of GSH synthesis. OAS synthesis from L-serine and acetyl-CoA is catalyzed by serin-*O*-acetyltransferase (SAT). Overproduction of mitochondrial SAT encoded by *TgSATm* of tiny wild mustard *T. goesingense* promoted accumulation of GSH in leaves of *A. thaliana*, providing increased tolerance to Ni, Co, Zn, and Cd,

attributed mainly to the acquired advantage of an improved antioxidative defense potential (Freeman and Salt 2007). In cysteine biosynthesis, inorganic sulfate after uptake is activated by ATP sulfurylase to form adenosine phosphosulfate (APS), which is subsequently reduced to free sulfide by APS reductase. In an extensive study measuring the effect of ATP sulfurylase overproduction on the accumulation of 12 metal and metalloid cations and oxyanions, Wangeline et al. (2004) observed that the expression of the *APS1* gene of *A. thaliana* in *B. juncea* markedly contributed to the metallotolerance in seedlings. Compared to WT controls, shoots of transgenic seedlings from the complex metalliferous media then contained higher levels of Cd (1.9 times), VO_4^{3-} (2.5 times), CrO_4^{2-} (1.5 times), WO_4^{2-} (1.7 times), and MoO_4^{2-} (1.4 times). The higher tolerance and accumulation of cations was attributed to the ATP sulfurylase-promoted increase in GSH levels (Pilon-Smits et al. 1999). The authors also suggested that enhanced accumulation of the metal oxyanions, as are sulfate analogues (Leustek 1996), was contributed by upregulation of sulfate uptake function to complement virtual sulfate starvation caused by the removal of free sulfate by the overproduced enzyme. Indeed, constitutive expression in *B. juncea* of *SHST1* gene encoding of a high-affinity plasma membrane sulfate transporter from pencil flower *Stylosanthes hamata* was later demonstrated promoting uptake of metal oxyanions in the same manner (Lindblom et al. 2006). Current research on the use of nicotianamine to promote metal uptake and translocation is largely focused on improving micronutrient (Zn, Fe) contents in crops (Johnson et al. 2011; Lee et al. 2011; Zheng et al. 2010). In a study relevant to phytoremediation, Kim et al. (2005) showed that introduction of barley nicotianamine synthase *HvNAS1* gene into *N. tabacum* rendered tobacco producing 5 times higher NA levels than control WT. Consistent with the translocation-promoting role of NA *in planta*, transgenic plants accumulated from serpentine soil by 1.3, 3.3, 2.1, and 4.0 times higher concentrations of Ni, Fe, Zn, and Mn than WT tobacco.

12.4.4 Plants Engineered to Produce Heterologous Metal-Binding Proteins

Overproduction of recombinant MTs to enhance metalloresistance and to support metal accumulation in plants has been the first strategy considered for the construction of phytoremediation plants (Misra and Gedamu 1989; Evans et al. 1992; Pan et al. 1994; Hasegawa et al. 1997; de Borne et al. 1998). This approach, applied in several laboratories, has resulted in different phenotypes. Although the constitutive expression of genes encoding mouse MT-1, human hMT-1A and h-MT-II, Chinese hamster MT-II, and yeast CUP1 in tobacco cabbage *Brassica oleracea* and *A. thaliana* markedly enhanced Cd resistance, the transgenic plants showed a 20–70 % reduction in metal accumulation in the shoots. On the other hand, production of CUP1 in *N. tabacum* grown in soil with 1,645 mg kg^{-1} Cu resulted

in threefold higher levels of Cu in leaves of transgenic plants compared to those from WT (Thomas et al. 2003). However, CUP1 tobacco did not show improved capacity to accumulate Cd. Both Cd tolerance and accumulation was improved in *N. tabacum* transformed with a fusion gene encoding HisCUP1, the CUP1 additionally modified with an N-terminal hexahistidine (His) extension (Macek et al. 2002; Pavlikova et al. 2004). In these plants, HisCUP represented 10 % of cellular cysteine-rich peptides involving glutathione and PCs (Křížková et al. 2007). Moreover, transgenic plants grown on sandy soil amended with 0.2 mg kg^{-1} Cd showed by 50 % reduced retention of Cd in roots and twofold higher levels of Cd in shoots than the control WT plants. Increased Cu accumulation was reported for roots, but not shoots, of *A. thaliana,* expressing the plant MT gene *PsMTA1* of pea *Pisum sativum* (Evans et al. 1992). In contrast, expression of *PsMTA1* in the white poplar *Populus alba* rendered Cu-tolerant plants, which translocated to shoots 3 times more Cu than did WT plants (Balestrazzi et al. 2009; Turchi et al. 2012). Some increase in uptake of Cu and Cd in shoots, but also higher retention of the metals in roots, was observed in *A. thaliana* producing CcMT1 of pigeonpea *Cajanus cajan* (Sekhar et al. 2011). Shoots of CcMT1 *Arabidopsis* accumulated by 50 % and 30 % higher concentrations of Cu and Cd, respectively. While most flax (*Linum usitatissimum*) show Cd-tolerant phenotype, they retain 70 % of accumulated Cd compartmentalized in roots (Bjelkova et al. 2011; Najmanova et al. 2012). The translocation of Cd to shoots has been improved in flax, which expressed high-affinity Cd-binding α-domain of mammalian MT1 isoform 1a (Vrbova et al. 2012). When tested in soils amended with Cd at 20 and 360 mg kg^{-1}, the mature transgenic flaxes contained in stems, respectively, 3.3- and 1.9-fold higher Cd levels than WT.

Transgenic plant *N. tabacum* was also constructed to demonstrate contribution of MTs to Hg tolerance and accumulation (Ruiz et al. 2011). When grown in hydroponic media with 15 μM Hg, transgenic tobacco producing mouse MT1 showed healthier growth and twofold Hg in leaves and stems than control WT plants. Periplasmic protein MerP is a component of bacterial Hg resistance, which is responsible for funneling metal ions to the uptake transporters MerT, MerC, or MerF (Silver and Phung 2005). When produced in *A. thaliana*, MerP got localized in the cell membrane and vesicles of plant cells (Hsieh et al. 2009). Unlike the WT control, MerP plants germinated on media with 12.5 μM Hg and accumulated 5.35 μg Hg g^{-1} of fresh seedling weight.

12.4.5 Modifications for Phytovolatilization of Mercury

Phytovolatilization of Hg and organomercurial compounds ($R\text{-}Hg^{+}$) involves the accumulation of metal species in plant cells and their subsequent conversion to volatile metallic Hg^{0}, which can be liberated to atmosphere through leaf evaporation. To this end, genetic determinants of widespread bacterial resistance to Hg and $R\text{-}Hg^{+}$ are employed, which involve *merA* encoding mercuric reductase, which

converts Hg to nontoxic volatile metallic Hg^0, and *merB* coding for organomercurial lyase, liberating Hg from R-Hg^+ (Silver and Phung 2005). The main advantage of phytovolatilization is the removal of Hg from a site without the need for plant harvesting and disposal. Although there could be some skepticism regarding the safety of such strategy, safety assessment studies on mercury phytovolatilization have indicated that the advantage of wide dispersion and dilution in the atmosphere and eventually to other environment components outweigh the potential risks (Lin et al. 2000; Moreno et al. 2005). Expression of *merA*, *merB*, or a combination of both, in *A. thaliana* (Bizily et al. 2003; Yang et al. 2003), *N. tabacum* (He et al. 2001; Ruiz et al. 2003, Haque et al. 2010), rice *Oryza sativa* (Heaton et al. 2003), saltmarsh cordgrass *Spartia alterniflora* (Czakó et al. 2006), yellow poplar *Liriodendron tulipifera tulipifera* (Rugh et al. 1998), and cottonwood *Populus deltoides* (Che et al. 2003; Lyyra et al. 2007), resulted in Hg and R-Hg^+-tolerant phenotypes (Table 12.2). To achieve efficient volatilization of mercury, use of modified versions of *merA* optimized for plant codon preferences (*merApe9* and *merA18*) were shown instrumental in achieving efficient production of MerA and pronounced mercury volatilization in *A. thaliana*, *N. tabacum*, and *L. tulipifera* (Rugh et al. 1998; He et al. 2001). While cytoplasmatic MerB allowed *A. thaliana* plants to grow at fivefolds higher methyl mercury concentrations compared to WT controls, the additional expression of *merApe9* further improved tolerance by a factor of 10 and promoted efficient phenyl mercury removal and Hg^0 volatilization from a model solution (Bizily et al. 2000). More than a 10-fold higher volatilization rate was further achieved by the targeting of MerB in the endoplasmatic reticulum (ER) of *merA*/*merB* double transformant (Bizily et al. 2003). The likely reason was that ER-localizing MerB exhibited more than a 20-times higher specific activity than in MerB plants with cytoplasmic MerB.

12.5 Genetic Engineering of Plant Symbionts

Several of the plant-associated bacteria and fungi have been reported to accelerate phytoremediation in metal-contaminated soils by promoting plant growth and health and play a significant role in accelerating phytoremediation (Miransari 2011; Rajkumar et al. 2012). In an aspect specific to plant–metal interaction, rhizosphere microbiota plays in its mutualistic associations with plants an important dual role in metal homeostasis: scavenging of metal micronutrients and their supply to the host plant; detoxification of excess essential metals and nonessential metal species as well. In general, the plant-associated bacteria migrate from the bulk soil to colonize the rhizosphere and roots of plants. Endophytic bacteria have been isolated from many different plant species as those colonizing the apoplast or symplast without causing negative effects on their host. Mycorrhizal fungi, especially species forming arbuscular mycorrhizae (e.g., an abundant *Glomus* spp.), develop symbiotic association with most of terrestrial plants. An original approach to modulate the heavy-metal accumulation capability in leguminous plants by

Table 12.2 Properties of plants genetically engineered for mercury volatilization

Overexpressed activity	Species	Transformed gene	Phenotype as compared to WT controls	References
Mercuric reductase	*A. thaliana*	Optimized *merA* of *E. coli* (*merApe9*)	2.5 times higher rate of Hg volatilization from hydroponic medium with 5 μM Hg (rate of 5 μg Hg^0 [g FW]$^{-1}$ min^{-1}). Germinating on media with 50–100 μM Hg (25 μM Hg is lethal to germination of WT seeds).	Rugh et al. (1998)
	N. tabacum	Optimized *merA* of *E. coli* (*merApe9*)	Increased rate of Hg volatilization from solution with 25 μM Hg: 8 times by roots (23.8 μg Hg^0 [g FW]$^{-1}$ min^{-1}), 3 times by leaves (6.9 μg Hg^0 g^{-1} min^{-1}), and 5 times by stem (4.1 μg Hg^0 g^{-1} min^{-1}). Germinating on media with 100–350 μM Hg (50 μM Hg is lethal to germination of WT seeds).	He et al. (2001)
	N. tabacum	*merA* of *E. coli*	Increased rate of Hg volatization from solution with 25 μM Hg after 3 days: 9 times by roots (467 μg Hg^0 [g FW]$^{-1}$), 8 times by leaves (131 μg Hg^0 g^{-1}), and 5 times by stem (92 μg Hg^0 g^{-1}). Transgenic roots resisted up to 140 μM Hg (WT did not survive beyond 20 μM Hg).	Haque et al. (2010)
	L. tulipifera	Optimized *merA* of *E. coli* (*merA18*)	Hg volatization from hydroponic media with 10 μM Hg improved 10 times (rate of 1.2 μg Hg^0 [g FW]$^{-1}$ per day). Germinating on media with 50 μM Hg (25 μM Hg is lethal to germination of WT seeds).	Rugh et al. (1998)
Organomercurial lyase plus mercuric reductase	*N. tabacum* (chloroplast)	*merB* and *merA* of *E. coli*	Doubled biomass yield with seedlings grown on medium with 400 μM phenyl-Hg^+.	Ruiz et al. (2003)
	N. tabacum (chloroplast)	Optimized *merB* of *E. coli* (*merBpe*) and *merApe9*	Seedlings volatized Hg from solution with 25 μM phenyl-Hg^+ at rate of 60 ng Hg^0 [g FW]$^{-1}$ min^{-1}. Germinating on media with 10 μM CH_3-Hg^+ (1 μM CH_3-Hg^+ is lethal to germination of WT seeds).	Bizily et al. (2000)
	S. alterniflora	Optimized *merB* of *E. coli* (*merBpe*) and *merApe9*	Callus culture can tolerate 500 μM Hg and 100 μM phenyl-Hg^+ (225 μM Hg or 50 μM phenyl-Hg^+ are lethal to WT callus).	Czakó et al. (2006)
	A. thaliana (MerB to endoplasmatic reticulum)	*merB* of *E. coli* plus *merApe9*	Seedlings volatized Hg from solution with 25 μM phenyl-Hg^+ at rate of 760 ng Hg^0 [g FW]$^{-1}$ min^{-1}.	Bizily et al. (2003)

engineering root-associated rhizobia was employed by Ike et al. (2007). Rhizobia establish a symbiotic relationship with leguminous plants and forms nitrogen fixing-nodule that contains more than 10^8 bacterial progenies. When PCS gene *AtPCS1* from *A. thaliana* along with a genetic fusion of four mammalian MT-coding sequences were expressed in *Mesorhizobium huakuii* subsp. *rengei* (strain B3), the natural capability of the bacterium to accumulate Cd from media containing 30 μM Cd increased by 25-fold. The colonization of Chinese milkvetch (*Astragalus sinicum*) with the B3 strain in rice-paddy soil containing 1 mg kg^{-1} Cd promoted uptake of the metal in roots, but not in nodules, by three times. Although the enhanced Cd accumulation phenotype of the roots was not accompanied by an increased metal translocation to the shoots, such a strategy would be useful in the rhizofiltration or transient phytostabilization of heavy metals in soil. The heavy metal-tolerant endophytes have been described from many hyperaccumulating plants (Rajkumar et al. 2012). In an attempt to investigate whether or not the introduction of endophytes engineered for the metal resistance would enhance phytoextraction of Ni, nickel tolerance *ncc-nre* genes were integrated into chromosomes of endophytic strains *Burkholderia cepacia* and *Herbaspirillum seropedicae* (Lodewyckx et al. 2001). Contrary to expectation, when modified strains were inoculated into host yellow lupin *Lupinus luteus* and ryegrass *Lolium perenne*, they apparently did not influence the growth of plants or cause an increased translocation of Ni *in planta*.

12.6 Conclusion and Future Prospect

Three different approaches are currently employed to develop transgenic plants suitable for phytoremediation. These include (1) increasing the number of metal transporters along with modulation of the specificity of the metal uptake system (2) enhancing intracellular ligand production and the efficiency of metal targeting into vacuoles to keep accumulated metal in a safe form without disturbing cellular processes and (3) biochemical transformation of metal volatile forms. A substantial experience has been gained, which helped to prove the suitability of heterologous and/or promoted intrinsic gene expression for the development of plants useful in phytoremediation. It is generally accepted that understanding of metal hyperaccumulation physiology and molecular basis underlying metal homeostasis and adaptation in hyperaccumulating species can greatly contribute to development of high biomass phytoremediation plants. Specifically, phytoremediation plants should be modified for effective long-distance metal translocation and repressed metal deposition in the roots and creation of artificial metal sinks in shoots. To this end, overproduction of highly mobile metal ligands such as nicotianamine by engineered plants or endophytes, manipulations to reduce transport into root vacuoles, and the shoot-specific expression of engineered cell-wall proteins with high-affinity binding sites for metal deposition in the apoplast of aboveground tissues could be instrumental. Successful phytoremediation of metal pollution may further involve

promoting mobilization of metals in soils. Efforts should thus be devoted to assess the effect that modifications for enhanced secretion of metal-complexing root exudates, ideally combined with implementation of the cognate metal-complex transport mechanism, would have on phytoextraction of soil metals. Conversion of immobile metals to their bioavailable forms in soils is largely dependent on the activity of soil microflora. Thus, modification of bacteria and fungi for secretion of protons and metal ligands can be also taken into account.

Genetically modified plants may endue remediation of heavy metal contamination with obvious benefits, yet some would question their techno-economic perspective and environmental safety. The best way to determine the true phytoremediation potential of genetically modified plants is by conducting field trials (Bañuelos et al. 2005, 2007; Van Huysen et al. 2004), which must be also designed to assess risks.

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Chapter 13
Phytoremediation Towards the Future: Focus on Bioavailable Contaminants

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13.1 Introduction

The term phytoremediation refers to a set of technologies that employ plants for soil, sediment and contaminated water remediation. Due to their simplicity, low cost and, above all, environmental benefits, phytotechnologies have raised considerable interest since 1990s for in situ remediation of contaminated soils. Of these techniques, metal phytoextraction is, at least theoretically, a brilliant strategy for the biological remediation of nonbiodegradable contaminants.

Phytoextraction and all other phytotechnologies have been extensively examined, discussed, and applied, and overall emerging framework has shown some positive results—along with several limitations, i.e., the need for further efforts to make them more efficient. In fact, there is a noticeable discrepancy between the number of scientific papers based on laboratory tests and the results achieved from concrete cleaning operations (Robinson et al. 2006). While the scientific community has found a challenging area of research, the field application of these technologies has encountered several difficulties that are often underestimated in theoretical studies. The results from experiments in hydroponics or in uncontaminated soils spiked with pollutants, although scientifically valid, do not reproduce the real conditions of contamination. Increasing concern derived from the differences between expectations resulting from the theoretical data and the practical realization of remediation have led to the conclusion that phytoextraction is not feasible in practice. This is due to the length of time required for remediation, and the difficulty in obtaining a high biomass production with high metal concentrations (Ernst 2005; McGrath et al. 2006; Robinson et al. 2006; Van Nevel et al. 2007).

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However, this conclusion is not completely true since some metals, such as nickel phytoextraction, have been employed with great efficiency (Ghaderian et al. 2007).

In some cases, technology also provides positive results with non-hyperaccumulator plants (Pedron et al. 2009; Koopmans et al. 2007). These successes derive, above all from the characteristics of contaminated soils, for instance, the pH that determines both the bioavailability of the contaminants and the conditions necessary for plant growth. Soil properties are the key to phytoextraction efficiency, but often they are not fully considered in the selection of the technology. Soils undergo physical, chemical, and biological reactions that continuously distribute metals among the various soil phases. Retention and release processes take place depending on each specific metal, soil properties, and time (Alexander 2000; Ehlers and Luthy 2003). Therefore, ability of the same plants to uptake metals is quite different among soils with different properties. Moreover, not all polluted soils and climate characteristics are suitable for all plant growth, including hyperaccumulators. Therefore a general scheme of phytoextraction that does not consider the specific properties of the contaminated soil may completely fail to predict the efficiency of the technology in field applications.

13.2 The Bioavailability of Contaminants: An Undervalued Aspect

The efficiency of all in situ technologies strictly depends on soil properties, which regulate the distribution of contaminants among the different soil phases. This is particularly important for phytoremediation, since plants absorb substances only if they are present in the soil liquid phase (soil solution). The evaluation of contaminant bioavailability is essential for the appropriate application of the technology. In soil, bioavailability is the result of complex mechanisms of mass transfer and absorption, which are affected by contaminant properties, the chemical and physical characteristics of the soil, and the biology of the organisms involved (National Research Council 2002). The transfer of heavy metals from the solid phase into soil solutions is fundamental. Only after being released in the aqueous phase, can a contaminant move freely towards the plant roots and be absorbed. Thus the metal speciation in soil is critical for phytoextraction under field conditions, while the concentration in the liquid phase is an essential parameter for the final success of remediation (Petruzzelli and Pedron 2006). In soils characterized by high contents of humic acids or the significant presence of clays, metals are strongly retained by these components, reducing the phytoextraction efficacy. In addition, stronger bonds between metals and soil surfaces correspond to the increasing time of residence of metals in soil, a reduction in bioavailability, and therefore a decrease in the efficiency of phytoextraction (Shelmerdine et al. 2009).

13.2.1 The Impact of Soil Properties

Before selecting a phytoextraction process, it is necessary to consider the specific characteristics of the soil at the contaminated site in order to evaluate how the soil properties will influence contaminant bioavailability and thus the final result of remediation in the field.

13.2.1.1 pH

In plant–soil–metal interactions, pH affects the uptake of metals in different ways for hyperaccumulator and nonaccumulator plants (Li et al. 2003; Chaney et al. 2005). pH is the most important parameter that determines the concentrations of metals in soil solutions by regulating precipitation–dissolution processes. pH values also regulate the specific adsorption and complexation of inorganics in the soil environment. Metal hydrolysis is also regulated by pH and beyond a threshold pH level (which is specific for each metal) these reactions drastically reduce the concentration of most metal ions in the soil pore water. At low pH levels, on the other hand, sorption processes are reduced due to the acid-catalyzed dissolution of oxides and their sorption sites, whereas complexation by organic matter tends to decrease with increasing acidity.

13.2.1.2 Clay Content

The influence of clay content on phytoremediation has been reported for specific species (Abdullah and Sarem 2010), but, in general, clay minerals regulate the amounts of metals in soil solutions. Ion exchange and specific adsorption are the mechanisms by which clay minerals adsorb metal ions from the soil liquid phase. This is done through the adsorption of hydroxyl ions followed by the attachment of the metal ion to the clay by linking it to the adsorbed hydroxyl ions or directly to sites created by proton removal. Highly selective sorption occurs at the mineral edges. However, notable differences exist between clay minerals in terms of their ability to retain heavy metals, which are more strongly adsorbed by kaolinite than montmorillonite. This is probably due to a higher amount of weakly acidic edge sites on kaolinite surfaces. In expandable clays (vermiculite and smectite), sorption essentially involves the interlayer spaces and is greater than in non-expandable clays such as kaolinite. The importance of clay minerals, and of soil texture, in determining the distribution of heavy metals between the solid and the liquid phases of soil has a direct consequence on the metal bioavailability of plants and phytoextraction efficiency.

13.2.1.3 Organic Matter Content

The efficiency of phytoextraction is often linked to humic content in contaminated media (Wanga et al. 2010). The organic matter of soils has a great influence on metal mobility and bioavailability due to the tendency of metals to bind with humic compounds in both the solid and solution phases in soil. The formation of soluble complexes with organic matter, in particular the fulvic fraction, is responsible for increasing the metal content of soil solutions. However, higher molecular weight humic acids can greatly reduce heavy metal bioavailability due to the strength of the linkages. Both complexation and adsorption mechanisms are involved in the linking of metals by organic matter, thus including inner-sphere reactions and ion exchanges (Pezzarossa and Petruzzelli 2001). Negatively charged functional groups (phenol, carboxyl, amino groups, etc.) are essential in metals that are retained by organic matter. The increase in these functional groups during humification increases the stability of metal organic complexes, which also show a greater stability at higher pH values.

13.2.1.4 Cation Exchange Capacity

The density of negative charges on the surfaces of soil colloids is determined by the type of clay and the amount of organic colloids present in the soil. The negative surface charges may be pH dependent or permanent. To maintain electro neutrality, they are balanced reversibly by equal amounts of cations from the soil solution. Weak electrostatic bonds link cations to soil surfaces, and heavy metals can easily substitute alkaline cations on these surfaces by exchange reactions. In addition, specific adsorption promotes the retention of heavy metals, also by partially covalent bonds, although major alkaline cations are present in soil solutions at much greater concentrations. This can drastically reduce the possibility of plants absorbing inorganic contaminants.

13.2.1.5 Redox Potential

Reduction–oxidation reactions in soils are controlled by redox potential (Eh). High levels of Eh are encountered in dry, well-aerated soils, while soils with a high content of organic matter or subject to waterlogging tend to have low Eh values. Plant-induced reductions of the redox potential and low Eh values can promote the solubility of some metals such as arsenic (Cherlatchka and Cambier 2000), thus increasing metal phytoextraction. This can be ascribed to the dissolution of Fe–Mn oxyhydroxides under reducing conditions, thus resulting in the release of adsorbed metals. However, under anaerobic conditions, the solubility of heavy metals could decrease when sulfides are formed from sulfates thus diminishing plant uptake.

13.2.1.6 Iron and Manganese Oxides

Hydrous Fe and Mn oxides are particularly effective in influencing metal solubility in relatively oxidizing conditions. They are important in reducing metal concentrations in soil solutions by both specific adsorption reactions and precipitation. Although Mn oxides are typically less abundant in soils than Fe oxides, they are particularly involved in sorption reactions with heavy metals. Mn oxides also adsorb heavy metals more strongly, thus reducing their mobility and thus reducing phytoextraction efficiency. Under reduced conditions, on the other hand, the dissolution of Fe and Mn oxides/hydroxides can release adsorbed Arsenic, and phytoextraction is promoted (Fitz and Wenzel 2002). When a chelating agent such as EDTA is used to increase metal solubility (Pb), this can also promote the dissolution of oxy-hydroxides, thus also promoting the uptake of different inorganic elements such as arsenic (Pedron et al. 2010).

13.2.1.7 Other Factors

There are a number of other factors that may affect the solubility of metals in soils and in turn phytoextraction efficiency. Temperature, which influences the decomposition of organic matter, can modify the mobilization of organometal complexes and consequently plant uptake. An increase in the ionic strength of soil solutions reduces the sorption of heavy metals by soil surfaces, due to the increased competition from alkaline metals (Petruzzelli and Pezzarossa 2003). Similar effects also derive from the simultaneous presence of many heavy metals in soil solutions, and these metals compete for the same sorption sites. This increases mobility in contaminated soils due to the saturation of adsorption sites. The living phase of soil is also of great importance in determining metal solubility, which is dependent to some extent both on microbial and on root activity. In the rhizosphere, microbial consortia are able to mobilize metals by changes in the rhizosphere pH. Plants can increase metal solubility following the release in the exudates both of protons, which increase the acidity, and of organic substances which act as complexing agents. Microbial biomass may promote the removal of heavy metals from soil solutions by precipitation as sulfides and by sorption processes on new available surfaces characterized by organic functional groups (Wenzel 2009).

13.3 Bioavailability with a View to Phytoextraction

Depending on the soil's properties, metals are distributed in soil in different pools of availability to plants. In phytoextraction, only metals in soil solutions will be available for plant uptake. This amount should be considered in terms of both intensity and capacity. Intensity identifies the concentration of metals in a soil

solution, while capacity is related to the ability of the soil to resupply metals in the soil solution following depletion due to plant uptake (Hough et al. 2005). The processes that determine bioavailability are the release of elements from the solid phase of soil and their uptake in soluble form by the root system of the plant. Bioavailable metal pools in soil decrease with time, due to both plant uptake and aging processes, which poses severe limitations to the amount of metals that can be removed by the technology. Both the theoretical modeling and the considerations deriving from the cases of application on a real scale show that phytoremediation is naturally limited by the considerably long time required, since it is a technique related to the growth cycles of plants. Decades of remediation would be necessary in many cases, which reduce the appeal of phytoremediation, especially if rapid results and a total removal of pollutants are required.

In order to increase the efficiency of phytoextraction, fertilizers can be used to enhance the productivity of selected plants, positive results have reported recently in the case of the boron-contaminated soils (Giansoldati et al. 2012). Amendments such as organic acids or synthetic chelators can be added to soil in order to facilitate desorption of metals from the solid phase and to increase, consequently, their solubility (assisted phytoextraction). However, the use of chelators able to form stable and water-soluble complexes with toxic metals can increase their concentrations in the soil solution for a long time and in excess of the translocation capacity of plants (Luo et al. 2005; Santos et al. 2006; Cao et al. 2007), being of potential concern for their leaching into the subsoil or into ground or surface waters. The use of natural low molecular weight organic acids such as citric, malic, oxalic, and tartaric acids and the natural amino acid, glutamic acid, which are characterized by a much lower toxicity and higher biodegradability, has been proposed as an alternative (Wu et al. 2004; Evangelou et al. 2006; Doumett et al. 2008). According to their rapid biodegradability, these ligands show a short persistence in soil (Evangelou et al. 2008). Repeated applications may therefore be suitable for maintaining metal bioavailability in soils high enough to support plant metal uptake.

Other promising possibilities consist in enriching the rhizosphere of plants with rhizobacteria that promote growth. The biogeochemistry of inorganic contaminants may be substantially influenced by the processes that happen in the rhizosphere. In the rhizosphere, while uptaking metals, roots induce changes in soil water transport and, by the exudation of proton, hydroxyl ions, and organic acids, can modify pH, redox conditions, and the chemical speciation of metals (Fitz and Wenzel 2002; Vetterlein et al. 2007; Wenzel 2009; Lin et al. 2010). Finally genetic engineering has made it possible to increase the tolerance and the accumulation of metals in species already characterized by a high production of biomass (Bizily et al. 2000; Meagher and Heaton 2005; Hussein et al. 2007). To sum up, in the case of heavy metal pollution, the application of phytoremediation on a large scale presents some problems and, in most cases, excellent results have not yet been achieved. In order to optimize the technique, research is moving in different directions. The use of genetically modified plants (Meagher et al. 2000) seems to offer important prospects, including economic benefits, and the addition of new agents that

mobilize metals to soil appears to increase the bioavailable amount without creating undesirable environmental side effects (Doumett et al. 2011). The design of plant microbial consortia, on the other hand, that would be able to modify the rhizosphere environment, could increase bioavailability and the uptake of heavy metals. In addition, the foliar treatment with phytohormones such as cytokinin increased the phytoextraction efficiency of crop plants in mercury-contaminated soil through the increase in plant biomass and evapotranspiration (Barbafieri and Tassi 2010; Cassina et al. 2012).

Another solution is to use plants to reduce only the fraction that is the most hazardous to the environment and human health: the mobile fractions of metals in soils (Fitz et al. 2003; Wenzel 2009). In this way, plants can be used to decrease bioavailable metals, while the cleanup time can be substantially shortened. With this approach based on the concept of bioavailable contaminant stripping (BCS) introduced by Hamon and McLaughlin (1999), an evaluation of the hazards of the residual fraction not removed by plants left to risk assessment procedures. This option is also supported by new legislation which no longer defines pollution on the basis of target concentrations, but according to the results derived from a site-specific risk analysis.

This remediation strategy originates from the intrinsic properties of the technology, whose applicability is strictly linked to the bioavailability of heavy metals. As previously highlighted, phytoextraction acts only on the amounts of metals that are, or may be, bioavailable. Nevertheless, most contaminated sites contain a residual fraction of metals, which are bound irreversibly to soil surfaces that phytoextraction cannot remove. The main criticism of the BCS method is that it is unknown how long it will take to reintegrate mobile metals in soil solutions, once the original soluble amount has been entirely or in part removed by plants. This problem can be overcome by enhanced bioavailable contaminant stripping (EBCS) (Petruzzelli et al. 2011, 2012), which evaluates this amount through the combined use of:

- Chemical extraction with a mobilizing agent, specific for each metal, capable of rapidly solubilizing the maximum possible amount of a metal, in order to rapidly simulate the slow release of metallic elements from the soil solid phase.
- Pot experiments in which the selected mobilizing agent is used as an additive (assisted phytoextraction), which in successive growing cycles must confirm the absence of the bioavailable fractions.

Thus this amount of mobilized metal can be considered to correspond to the maximum potentially available, which can be removed by plants in one or more cycles of growth. The rate of resupply to the depletion of metals in a soil solution depends on metal pool speciation in the solid phase, where most metals are irreversibly linked (Lehto et al. 2006). The characterization of available pools and the ability to resupply metals from less available pools is essential to support any decision to apply this technology in the field. The use of pot experiments in addition to chemical extraction can help to evaluate whether there may be unpredictable events that hinder plant growth in contaminated soil or whether the plant roots are not able to access the metals in soil, for instance, due to poor physical

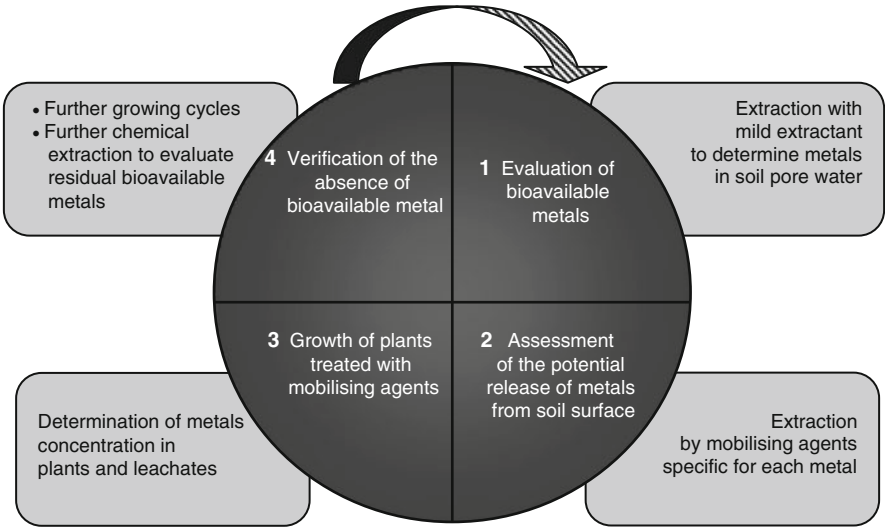

Fig. 13.1 Enhanced bioavailable contaminant stripping scheme

quality, which is not uncommon at contaminated sites. In addition, the plant growth may highlight the root-induced changes affecting metal bioavailability (Hinsinger and Courchesne 2008).

The EBCS approach shown in Fig. 13.1 can be subdivided into four steps:

1. Evaluation of the metal in a potentially bioavailable form. Soluble or easily solubilizable amounts are identified (i.e., the exchangeable species).
2. Determination of the total amount of long-term extractable metal over time. This step is performed making use of metal-specific mobilizing agents. Since the action of the mobilizing agent is much greater than any natural process, the amount determined in this step can be considered (based on a precautionary approach) as the maximum quantity of metals available to plants.
3. Growth of plants in pot experiments, in order to select the most efficient species on the basis of their ability to take up both the original and the newly created available fractions, which are brought into solution by the specific mobilizing agent. Control of the possible presence of metals in the leachates from pot trials.
4. After harvesting, further cycles of plant growth are performed on the same soils, with and without the addition of the specific mobilizing agent, in order to control the absence of residual metals in bioavailable forms. An extraction with the specific additive is carried out on soil to verify the absence of mobile chemical forms of the metallic element. When the metal concentration in plants is negligible and no amount of metal can be extracted from the soil by the specific mobilizing agent, the residual concentration of metal can be considered to be permanently unavailable.

13.4 A Case Study of Mercury-Contaminated Soil

Experiments were conducted to determine the possible utilization of EBCS in mercury-contaminated soil. The soil derived from an industrial site located in northern Italy. Soil samples were air dried and ground to pass through a 2-mm sieve before laboratory analysis. All analytical determinations were carried out according to the methods of soil analysis (Sparks 1998). Soil was characterized by: pH 7.1, organic matter 1.1 %, and cation exchange capacity 17.3 cmol(+) kg^{-1}. The texture was sand 58.4 %, clay 22.5 %, and silt 19.1 %. Mercury was the only contaminant. The total Hg concentration was 26.7 mg kg^{-1}.

In this work, the scheme of the EBCS approach consisted in the following steps:

1. Evaluation of the original Hg bioavailable soil fraction by the use of the sequential extraction with H_2O and NH_4Cl (Millán et al. 2006).
2. Evaluation of the long-term potential Hg release from soil surfaces with time. This step was performed by extraction with a specific solubilizing agent: ammonium thiosulphate $(NH_4)_2S_2O_3$ (Moreno et al. 2004, 2005; Pedron et al. 2011).
3. Selection of plant species and evaluation of removable mercury. Experiments were carried out at mesocosm scale, selecting the most efficient species.
4. Assessment of the existence of a residual bioavailable fraction in soil by both chemical extractions and further plant growing cycles. When the metal concentration in plants is negligible and no available fraction can be further extracted from soil by $(NH_4)_2S_2O_3$, the residual concentration of the metal in soil can be considered not bioavailable and can be safely left in soil.

13.4.1 Experimental Procedure

13.4.1.1 Soil Sequential Extraction

The mercury available fractions were determined by a two-step sequential extraction procedure (Millán et al. 2006) with H_2O and NH_4Cl. In the first step, 0.5 g of soil was treated with 25 mL of deionized water at pH 7.0 for 1 h at room temperature and the soil residue from water extraction was treated with 25 mL of ammonium chloride 1 M at pH 7.0 for 1 h at room temperature. Mercury concentration was determined in the surfactants from both extractions. The total available fraction was calculated as the sum of the water-soluble fraction and the exchangeable fraction. The long-term potential release from the soil solid phase was determined by 0.27 M ammonium thiosulphate extraction at pH 5.0 with a ratio soil/extractant 1:20.

13.4.1.2 Mesocosm Experiments

The trials were carried out at mesocosm scale in a greenhouse where the temperature was kept between 18 °C and 26 °C. Mesocosms were polypropylene containers

(height 30 cm, volume 8.15 L) that are arranged to collect leachates by a hole in the bottom connected to a plastic tank with a PVC tube. Two plant species were selected: *Brassica juncea* var. scala and *Helianthus annuus* var. paola. Coarser materials (>2 cm) were eliminated from soil before filling the mesocosms. The amount of soil per pot was 5 kg. Plants were sowed using 0.5 g of seeds for *B. juncea* and nine seeds for *H. annuus*. During the growing period, plants were watered daily with deionized water. Treatment with mobilizing Hg additive started 45 days after sowing, with the same solution used for Hg extraction, 0.27 M ammonium thiosulphate. The solution was added to mesocosms by splitting the total dose, 250 mL in 5 consecutive days to avoid or at least to minimize possible toxic effects on plant species. Three replicates of treated mesocosms (TS) were prepared, with controls (CT) (untreated soil) run simultaneously. Experiments lasted 60 days. Plants were harvested 15 days after additive treatment. Aerial parts were separated from the roots and all samples were washed with deionized water. The roots were subjected also to a washing in an ultrasound bath (Branson Sonifier 250 ultrasonic processor) for 10 min to eliminate soil particles that could have remained on root surfaces. Vegetal samples were left in a ventilated oven at a temperature of 40 °C until a constant weight was obtained. The dry mass of shoots and roots was gravimetrically determined. Materials were grinded and homogenized by the use of Knife Mill Grindomix GM 300 Retsch for analysis.

13.4.2 Results from EBCS

Step 1. Among the several reagents used for evaluating the bioavailable Hg fractions, the sequential extraction with H_2O and NH_4Cl was selected (Millán et al. 2006) since it properly individuates the amounts in the Hg soil solution (H_2O) or easily solubilizable (NH_4Cl) available for plant uptake. The analysis on extracts from the sequential extraction procedure showed that Hg soluble and exchangeable fractions represented a very low portion of the total concentration, 2.6 and 9.6 μg kg^{-1}, respectively. The sum of these two fractions was considered the "total available mercury" that can be immediately uptaken by plants.

Step 2. To assess the potential ability of the soil to replenish the available metal pool over time, extraction with a highly specific Hg-mobilizing agent, 0.27 M ammonium thiosulphate, was performed. The action of this extractant is much stronger than any natural process, and the amount released in this step can be safely considered as the maximum possible amount of metal available to plants. Ammonium thiosulphate extracted, as a mean, 0.12 mg kg^{-1} Hg before plant growth. A further extraction on the residual soil showed negligible amount of Hg below the detection limit. This extraction gives an estimate of the likely long-term bioavailable Hg.

Step 3. According to EBCS scheme, the extractant 0.27 M ammonium thiosulphate used to evaluate the release of mercury in the long term has been added to soil. In this way, a new bioavailable pool is created from which plants can

Table 13.1 Percentage of bioavailable fraction removal from different plant species in the first growing cycle

		Shoots (%)	Roots (%)
Brassica juncea	CT	19.3	10.8
	TS	73.7	22.9
Helianthus annuus	CT	13.4	30.8
	TS	26.0	69.8

uptake larger amounts of the contaminant. The efficiency of plant removal was determined by the ratio between total accumulation and total available mercury in soil. For both species, efficiency was higher than 95 % Table 13.1.

For controls, the percentage was calculated with respect to the amount of mercury extractable according to Millán et al. (2006), while for treated soil the percentage was determined with respect to the amount of Hg extractable by thiosulfate 0.27 mol L^{-1}. As a matter of fact, there was a slight increase in biomass production probably due to the fertilizing effect of ammonium thiosulphate. In the growth period of 60 days in the untreated soils, the mean values of the aerial biomass were 10.4 and 30.2 g DW for *B. juncea* and *H. annuus*, respectively. After ammonium thiosulphate treatment, the obtained results were 16.1 and 34.5 g in the case of *B. juncea* and *H. annuus*, respectively. Root biomass was not affected by treatment with mean values of 1.5 g DW for *B. juncea* and 2.8 g DW for *H. annuus* both in CT and TS soil. The results obtained for original (CT) and thiosulphate treated (TS) soils are reported in Fig. 13.2.

The addition of the mobilizing agent promoted Hg uptake by plants of both species. The data (Fig.13.2) clearly show that the uptake of the plants was dependent on Hg concentration in the soil solution that determines the metal bioavailability. The mean Hg concentrations in plants grown in the original soil were 2.20 and 32.9 mg kg^{-1} for aerial and root portions of *B. juncea* and 0.80 and 14.0 mg kg^{-1} in the case of *H. annuus*. The addition of TS solubilized greater quantities of Hg; this in turn increased the Hg uptake by plants. For *B. juncea* the mean Hg concentrations increased to 29.1 and 74.6 mg kg^{-1} in the aerial and root portions, respectively, while for *H. annuus* results of Hg concentration were 10.5 and 73.4 mg kg^{-1}. The increase in Hg concentrations in plants after TS addition has been reported also in different soils and plants (Moreno et al. 2004, 2005; Pedron et al. 2011). The lower concentrations of Hg in shoots reflect the defense mechanisms of plants that store the toxic metal in the root portion (Moreno et al. 2004, 2005). At the end of the experiments, the control analysis of leachates showed negligible Hg concentration, below the detection limit (2 μg L^{-1}), in any mesocosms.

The time required to achieve reduction of bioavailable concentration required in soil can be calculated by

$$t = \frac{W_s \cdot \Delta C}{C_v \cdot B}, \tag{13.1}$$

where t is time (years), W_s weight of soil (kg), and ΔC the decrease of metal concentration necessary to achieve the remediation target (mg kg^{-1}), i.e.,

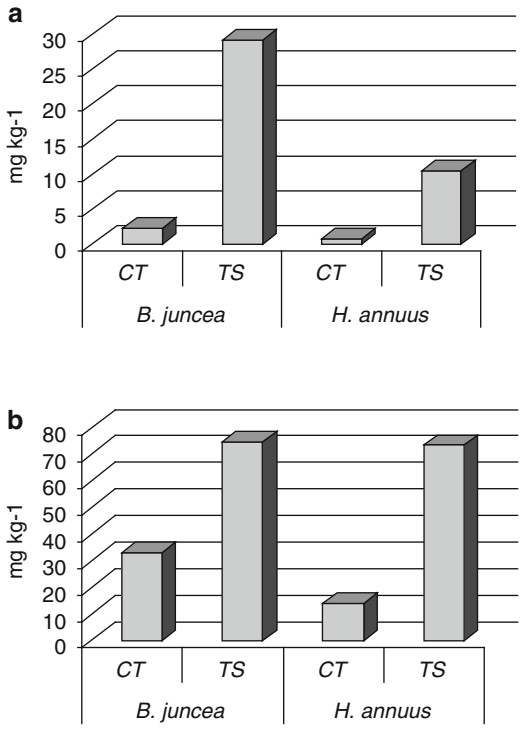

Fig. 13.2 Hg concentrations in the aerial part (**a**) and roots (**b**) of *Brassica juncea* and *Helianthus annuus* grown in control (CT) and thiosulphate-treated soil (TS)

elimination of the bioavailable fractions. C_v is the concentration of metal in plants (mg kg^{-1}) and B is the annual biomass production per mesocosm (kg per year). Both C_v and B depend on the soil characteristics and bioavailable forms of metals. These are the two essential parameters that determine the applicability of plant-based remediation. Considering one growing cycle per year, and inserting the specific values of this study in (13.1), the resultant time required is 1 year.

Step 4. To confirm the removal of all the bioavailable Hg, a second growing cycle (without any further treatment) using the same plants was carried out. Results showed a negligible uptake of the metal. Mesocosms were left to rest for 1 year and then they were reseeded. The new trials were prepared with the following scheme: some pots were sown with the same type of plants used in the past; in others, plant species were reversed, sowing *B. juncea* in pots where *H. annuus* grew in previous experiments and vice versa. At the end of the experiment, plants were collected and analyzed. The results showed that also in this case Hg concentrations in plant sample were below the detection limit. The soil extraction with TS did not extract Hg amount over the detection limit. This confirms that the entire Hg bioavailable portion has been removed and that new equilibria in the soil with subsequent release of bioavailable Hg were not created. Clearly these results are site specific and it is possible that in different contaminated soils more than one growth growing cycle would be necessary to eliminate all the bioavailable metal fractions. Throughout the

EBCS procedure, the potential gaseous loss of Hg(0) has been controlled (Mercury tracker 3000IP Mercury Instruments GMBH) and the values were negligible. When used at field scale, a monitoring program should be planned to control the absence of transport of mercury in the deeper soil layer.

Since the natural processes of depletion and accumulation are not quantitatively determinable in the short term, according to a precautionary principle we have modified the BCS remediation approach by adding a new step, in which mercury bioavailability was enhanced with the use of a mobilizing agent. In this way, the amount of Hg releasable over time is considered. This amount is largely greater than the one deriving from natural processes, and thus represents all the possible available Hg that can be removed by plants (remediation target), while the remaining Hg in soil can be safely considered unavailable. This hypothesis was supported by results from the second growing cycle. If we move from current definition of remedial targets based on total metal concentrations, EBCS appears promising, since it removes the most dangerous metal forms while substantially shortening the cleanup time, with an elevated security. After EBCS, the residue metal in soil will remain unavailable over time, since it was not released in mobile forms even with the use of strong mobilizing agents.

13.5 Concluding Remarks

The concept of soil quality has evolved in response to the increased demand for a sustainable land use. It has been recognized that soil is essential for the environment, and new strategies must be defined for soil protection. Contamination is one of the most important threats to soil quality and contaminated soils must be cleaned up. However in remediation procedures, soil quality has often been considered only marginally. The new trends in remediation strategies "Green Remediation" have recovered the importance of soil quality. Green remediation technologies are rapidly expanding in the world to reduce overall environmental impact of cleanup. Green remediation is a completely new strategy of remediation that consider environmental impacts of remediation activities at every stage of the remedial process in order to maximize the net environmental benefit of a cleanup. Considerations include selection of a remedy, energy requirements, efficiency of on-site activities, and reduction of impacts on surrounding areas (USEPA 2008a). Among the core elements of green remediation there is the aim to minimize the bioavailability of contaminants by the use of minimally invasive technologies to reduce soil and habitat disturbance. In this frame, the use of a solar-driven biological technology such as phytoremediation as primary remedy or finishing step is strongly recommended.

Considerable future research for the improvement of phytoremediation is still necessary. Multidisciplinary efforts are needed to combine plant biology, soil chemistry, and microbiology, as well as agricultural practices, but high efficiency of phytoextraction will be obtained only if bioavailable contaminant is in contact

with roots. The hypothesis that one of the possible future trends of phytoextraction should be the removal of the bioavailable contaminants has recently received renewed and increasing interest (Fitz et al. 2003; Van Nevel et al. 2007; Koopmans et al. 2007). This approach can be safely applied if the soil ability to replenish the bioavailable pools in the long term is considered as in the case of EBCS procedure. In this frame phytoextraction can be evaluated and selected to minimize the mobile and bioavailable fractions of contaminants, while improving soil quality. This strategy should be carefully checked using an appropriate risk that incorporates specific considerations of bioavailability (USEPA 2008a, b) to assess the potential risks arising from the presence of any residual quantity of metals, even if inert, in a contaminated site. The field scale applicability of phytoremediation is constrained the long time required to achieve the remediation target, however, if the focus of the technology is on the bioavailable contaminant fractions the time for remediation is reduced. The technology does not remove, in general, great amounts of contaminants, but plants are able to uptake the most environmentally significant fractions. The technology is not invasive and it is able to improve the soil quality at the end of the treatment. The selection of phytoextraction can avoid the excavation and landfilling of soil, a practice that in few hours destroy what the nature has created in hundreds of years.

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Index

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