Review

Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic

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Abstract

Due to their immutable nature, metals are a group of pollutants of much concern. As a result of human activities such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, etc., metal pollution has become one of the most serious environmental problems today. Phytoremediation, an emerging cost-effective, non-intrusive, and aesthetically pleasing technology, that uses the remarkable ability of plants to concentrate elements and compounds from the environment and to metabolize various molecules in their tissues, appears very promising for the removal of pollutants from the environment. Within this field of phytoremediation, the utilization of plants to transport and concentrate metals from the soil into the harvestable parts of roots and above-ground shoots, i.e., phytoextraction, may be, at present, approaching commercialization. Improvement of the capacity of plants to tolerate and accumulate metals by genetic engineering should open up new possibilities for phytoremediation. The lack of understanding pertaining to metal uptake and translocation mechanisms, enhancement amendments, and external effects of phytoremediation is hindering its full scale application. Due to its great potential as a viable alternative to traditional contaminated land remediation methods, phytoremediation is currently an exciting area of active research.

1. Environmental metal pollution and phytoremediation

Soil pollution has recently been attracting considerable public attention since the magnitude of the problem in our soils calls for immediate action (Garbisu & Alkorta 2003). As a result of human activities such as mining and smelting of metalliferous, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, etc., metal pollution has become one of the most serious environmental problems today. Due to their immutable nature, metals are a group of pollutants of much concern. In fact, although several metals are essential for biological systems and must be present within a certain concentration range (Garbisu & Alkorta 2003), at high concentrations, metals can act in a deleterious manner by blocking essential functional groups, displacing other metal ions, or modifying the active conformation of biological molecules (Collins & Stotzky 1989). Metal toxicity for living organisms involves oxidative and/or genotoxic mechanisms (Briat & Lebrun 1999).

Based on their chemical and physical properties, three different molecular mechanisms of heavy metal toxicity can be distinguished: (i) production of reactive species by autooxidation and Fenton reaction (Fe, Cu), (ii) blocking of essential functional groups in biomolecules (Cd, Hg), and (iii) displacement of essential metal ions from biomolecules (Schutzendubel & Polle 2002).

Metal-contaminated soils are notoriously hard to remediate. Current technologies resort to soil excavation and either landfilling or soil washing followed by physical or chemical separation of the contaminants. Although highly variable and dependent on the contaminants of concern, soil properties, site conditions, and so on, the usually enormous costs associated with the removal of metals from soils by means of traditional physicochemical methods explain why most companies tend to ignore the problem. Due to the fact that very often large areas are affected by heavy metal contamination, a removal is certainly difficult. Therefore, some methods are developed to keep the metals in the soil but reduce the risks related to this presence (e.g., by decreasing bioavailability by *in situ* immobilisation processes) (Diels et al. 2002). One way to facilitate such immobilisation is by altering the physicochemical properties of the metal-soil complex by introducing a multipurpose anion, such as phosphate, that enhances metal adsorption via anion-induced negative charge and metal precipitation (Bolan et al. 2003a).

Heavy metals cannot be destroyed biologically (no "degradation", change in the nuclear structure of the element, occurs) but are only transformed from one oxidation state or organic complex to another (Garbisu & Alkorta 2001). Although microorganisms that use metals as terminal electron acceptors or reduce them as part of a detoxification mechanism can be used for metal remediation (Garbisu & Alkorta 1997), when considering the remediation of metal-polluted soil, metal-accumulating plants offer numerous advantages over microbial processes since plants can actually extract metals from the polluted soils, theoretically rendering them clean (metal-free) (Garbisu & Alkorta 2001; Garbisu et al. 2002).

Phytoremediation, the use of plants to extract, sequester, and/or detoxify pollutants, has been reported to be an effective, non-intrusive, inexpensive, aesthetically pleasing, socially accepted technology to remediate polluted soils (Alkorta & Garbisu 2001; Weber et al. 2001; Garbisu et al. 2002). Phytoremediation is widely viewed as the ecologically responsible alternative to the environmentally destructive physical remediation methods currently practiced (Meagher 2000). The US phytoremediation market is expected to expand more than ten-fold between 1998 and 2005, to over $214 million (Evans 2002).

In the last few years, some excellent reviews have been published focusing on different aspects of phytoremediation (Salt et al. 1995a, 1998; Chaney et al. 1997; Raskin et al. 1997; Chaudhry et al. 1998; Wenzel et al. 1999; Meagher 2000; Navari-Izzo & Quartacci 2001; Lasat 2002; McGrath et al. 2002; McGrath & Zhao 2003; McIntyre 2003; Singh et al. 2003). In any case, and in contrast to its many positive aspects, phytoremediation does have certain disadvantages and limitations (Table 1).

Table 1. Advantages and limitations of the phytoremediation technology

Advantages	Limitations
Applicable to a wide variety of inorganic and organic contaminants.	Limited by depth (roots) and solubility and availability of the contaminant.
Reduces the amount of waste going to landfills.	Although faster than natural attenuation, it requires long time periods (several years).
Does not require expensive equipment or highly specialized personnel.	Restricted to sites with low contaminant concentration.
It can be applied *in situ*. Reduces soil disturbance and the spread of contaminants.	Plant biomass from phytoextraction requires proper disposal as hazardous waste.
Early estimates of the costs indicate that phytoremediation is cheaper than conventional remediation methods.	Climate and season dependent. It can also lose its effectiveness when damage occurs to the vegetation from disease or pests.
Easy to implement and maintain. Plants are a cheap and renewable resource, easily available.	Introduction of inappropriate or invasive plant species should be avoided (non-native species may affect biodiversity).
Environmentally friendly, aesthetically pleasing, socially accepted, low-tech alternative.	Contaminants may be transferred to another medium, the environment, and/or the food chain.
Less noisy than other remediation methods. Actually, trees may reduce noise from industrial activities.	Amendments and cultivation practices may have negative consequences on contaminant mobility.

Table 2. Categories of phytoremediation

Term	Definition
Phytoextraction	The use of plants to remove pollutants (mostly, metals) from soils.
Phytofiltration	The use of plants roots (rhizofiltration) or seedlings (blastofiltration) to absorb or adsorb pollutants (mostly, metals) from water.
Phytostabilization	The use of plants to reduce the bioavailability of pollutants in the environment.
Phytovolatilization	The use of plants to volatilize pollutants.
Phytodegradation Phytotransformation	The use of plants to degrade organic pollutants
Phytostimulation Enhanced rhizosphere degradation Rhizodegradation Plant-assisted bioremediation Plant-asssisted degradation Plant-aided *in situ* biodegradation	The use of plant roots in conjunction with their rhizospheric microorganisms to remediate soils contaminated with organics.

Within the field of phytoremediation, different categories have been defined such as, among others, phytoextraction, phytofiltration (rhizofiltration, blastofiltration), phytostabilization, phytovolatilization, phytodegradation (phytotransformation), plant-assisted bioremediation (plant-assisted degradation, plant-aided *in situ* biodegradation, phytostimulation, enhanced rhizosphere degradation, rhizodegradation), etc. (Table 2).

Plants for phytoextraction, i.e., metal removal from soil, should have the following characteristics: (i) tolerant to high levels of the metal, (ii) accumulate reasonably high levels of the metal, (iii) rapid growth rate, (iv) produce reasonably high biomass in the field, and (v) profuse root system (Garbisu et al. 2002).

The idea of using plants to remediate metal polluted soils came from the discovery of "hyperaccumulators" (Table 3), defined as plants, often endemic to naturally mineralized soils, that accumulate high concentrations of metals in their foliage (Baker & Brooks 1989; Raskin et al. 1997; Brooks 1998). In fact, plants growing on metalliferous soils can be grouped into three categories according to Baker (1981): (i) excluders, where metal concentrations in the shoot are maintained, up to a critical value, at a low level across a wide range of soil concentration; (ii) accumulators, where metals are concentrated in above-ground plant parts from low to high soil concentrations; and (iii) indicators, where internal concentration reflects external levels (McGrath et al. 2002). The

criterion for defining Ni hyperaccumulation is 1000 μg Ni g^{-1} on a dry leaf basis (Brooks et al. 1977), whereas for Zn and Mn the threshold is 10,000 μg g^{-1} and for Cd 100 μg Cd g^{-1}. Finally, the criterion for Co, Cu, Pb and Se hyperaccumulation is also 1,000 μg g^{-1} in shoot dry matter (Brooks 1998; Baker et al. 2000; McGrath et al. 2002). In general terms, metal concentrations in hyperaccumulators are about 100–1000-fold higher than those found in normal plants growing on soils with background metal concentrations, and about 10–100-fold higher than most other plants growing on metal-comtaminated soils (McGrath et al. 2002). Hyperaccumulators are also characterized by a shoot-to-root metal concentration ratio of >1 (i.e., hyperaccumulator plants show a highly efficient transport of metals from roots to shoots), whereas non-hyperaccumulators usually have higher metal concentrations in roots than in shoots (Baker 1981; Gabbrielli et al. 1990; Homer

Table 3. Examples of hyperaccumulators

Metal	Species
Zinc (Zn)	*T. caerulescens*
Cadmium (Cd)	*T. caerulescens*
Nickel (Ni)	*Berkheya coddii*
Selenium (Se)	*Astragalus racemosa*
Thallium (Tl)	*Iberis intermedia*
Copper (Cu)	*Ipomoea alpina*
Cobalt (Co)	*Haumaniastrum robertii*
Arsenic (As)	*P. vittata*

et al. 1991; Baker et al. 1994a,b; Brown et al. 1995; Krämer et al. 1996; Shen et al. 1997; Zhao et al. 2000; McGrath et al. 2002).

Hyperaccumulation of heavy metal ions is indeed a striking phenomenon exhibited by <0.2% of angiosperms (Baker & Whiting 2002). Most recently, a plethora of papers are being published in an attempt to dissect the mechanisms of metal uptake, transport and accumulation, both at the physiological and molecular level (Baker & Whiting 2002). The "model" hyperaccumulator *Thlaspi caerulescens* has been much screened in the search for new and more extreme ecotypes (McGrath et al. 2001; Lombi et al. 2002). Our understanding of the internal processes that confer the hyperaccumulation phenotype is advancing in leaps and bounds, and the mechanisms of transport, tolerance and sequestration in some species, at least in the genera *Thlaspi and Alyssum,* are partially elucidated (Lasat 2002).

Trees have also been considered for phytoremediation of heavy metal-contaminated land, with willow and poplar being promising candidates, among others, in this respect (Pulford & Watson 2003). According to some authors, trees potentially are the lowest-cost plant type to use for phytoremediation (Stomp et al. 1994). Many trees can grow on land of marginal quality, have massive root systems, and their above-ground biomass can be harvested with subsequent resprouting without disturbance of the site (Stomp et al. 1994).

Following the harvest of metal-enriched plants, the weight and volume of the contaminated material can be further reduced by ashing or composting (Garbisu & Alkorta 2001; Garbisu et al. 2002). Metal-enriched plants can be disposed of as hazardous material or, if economically feasible, used for metal recovery (Salt et al. 1998). Recently, some studies have reported on the utilization of pyrolysis to separate heavy metals from hyperaccumulators (Koppolu & Clements 2003).

Although plants acquire essential minerals such as Fe, Cu, Ni, Zn and Se from the soil, for reasons that are not yet clear, they also have the ability to acquire and detoxify non-essential elements such as As, Cd, Cr and Pb (Salt et al. 2002). Certain themes in the physiology and biochemistry of trace element accumulation by plants appear common (Salt et al. 2002).

Most phytoremediation studies have considered metal extraction efficiency in relation to metal concentration of bulk soil samples or metal concentration of the soil solution, but little is known about the effect of various metal-bearing solids on metal extraction by hyperaccumulators. In fact, it has been shown that it is essential to consider the nature of the metal-bearing solids to better predict the efficiency of plant extraction (Dahmani-Muller et al. 2001). Besides, it is also important to consider that metal bioavailability changes between the bulk soil and the rhizosphere, the latter being a microbiosphere which has quite different chemical, physical and biological properties from bulk soils (Wang et al. 2002b). In this respect, recently, it has been reported that root growth is a more sensitive endpoint of metal availability than chlorophyll assays (Morgan et al. 2002). In order to improve phytoremediation of heavy metal polluted sites, the speciation and bioavailability of the metals in the soil, the role of plant-associated soil microorganisms and fungi in phytoremediation, and that of plants have to be elucidated (Kamnev & van der Lelie 2000).

Phytoremediation has been used in mined soil restoration, since these soils are sources of air and water pollution, by means of phytostabilization and phytoextraction techniques to stabilize toxic mine spoils and remove toxic metals from the spoils, respectively (Wong 2003).

Some higher plant species have developed heavy metal tolerance strategies which enable them to survive and reproduce in highly-metal contaminated soils. Dahmani-Muller et al. (2000) investigated metal uptake and accumulation strategies of two absolute metallophyte species and one pseudometallophyte. In the former two species, real hyperaccumulation in the leaves as well as metal immobilisation in roots and/or a detoxification mechanism by leaf fall were found as possible strategies to deal with the high metal concentrations. By contrast, the strategy of the pseudometallophyte, i.e., *Agrostis tenuis*, presented a significant metal immobilisation by the roots.

Most plants have mycorrhizal fungi associated with them, providing their hosts with an increased capacity to absorb water and nutrients from the soil. The formation and function of mycorrhizal relationships are affected by anthropogenic stressors including metals (Entry et al. 2002). Arbuscular mycorrhizal fungi are of interest for their reported roles in alleviation of diverse
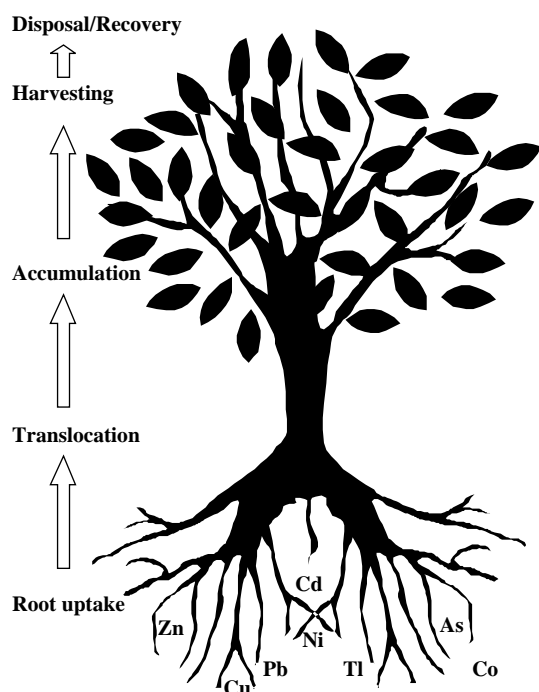
Figure 1. Phytoextraction of metals.

soil-associated plant stressors, including those induced by metals, so it has been claimed that the evaluation of the efficacy of plant-mycorrhizal associations to remediate metal-polluted soils deserves increased attention (Entry et al. 2002). In addition, phytoextraction practices, e.g., the choice of plant species and soil amendments, may have a great influence on the quantity and species composition of glomalean propagules as well as on arbuscular mycorrhizal fungi functioning during long-term metal-remediation treatments (Pawlowska et al. 2000).

A unique testing system, the target-neighbour method, has been described to allow evaluation of how planting density influences metal uptake, so that the information needed to manipulate plant density for optimization of metal removal could be obtained (Shann 1995).

Finally, most recently, phytoremediation has been combined with electrokinetic remediation, applying a constant voltage of 30 V across the soil, concluding that the combination of both techniques represents a very promising approach to the decontamination of metal polluted soils (O′Connor et al. 2003).

2. New findings on the phytoextraction of some of the most relevant environmentally toxic heavy metals (zinc, cadmium, lead) and metalloids (arsenic)

This section is divided into four different sub-headings. The first three correspond to three of the most environmentally relevant heavy metals, i.e., Zn, Cd, and Pb. The fourth sub-heading deals with As, a well-known toxic metalloid. It is important to emphasize here that very often information regarding one metal appears under a different, apparently wrong, sub-heading. Since many of the reviewed publications deal with more than one metal at the same time, it has been preferred to present them as part of the same research, despite the fact that section structure could not be maintained as desired.

2.1. *Zinc*

Zinc and Cd are ubiquitous pollutants that tend to occur together at many contaminated sites. While Zn is often phytotoxic, Cd rarely inhibits plant growth. In *T. caerulescens*, an integrated molecular and physiological investigation of the fundamental mechanisms of heavy metal accumulation was conducted (Pence et al. 2000). A metal transporter cDNA, *znt1* (expressed at very high levels in roots and shoots of this plant), was cloned from *T. caerulescens* through functional complementation in yeast and was shown to mediate high-affinity Zn uptake as well as low affinity Cd uptake. Alteration in the regulation of *znt1* gene expression by plant Zn status results in the over-expression of this transporter and in increased Zn influx in roots, even when intracellular Zn levels are high. Thus, specific alterations in Zn-responsive elements (e.g., transcriptional activators) possibly play an important role in Zn hyperaccumulation in *T. caerulescens* (Pence et al. 2000). In this respect, Lasat et al. (1998) found that the enhanced root-to-shoot Zn transport in *T. caerulescens* was, at least partly, achieved through an altered Zn compartmentation in the root symplasm, which reduces Zn sequestration in root vacuoles. A further step at elucidating the mechanisms underlying Zn hyperaccumulation was given thanks to the cloning of metal transporter genes encoding putative vacuolar ion transport proteins in *T. caerulescens* (Assunçáo et al. 2001).

In fact, ZTP1 (a transporter belonging to the cation-efflux family) has been found to be highly expressed in T. caerulescens, predominantly in leaves (Assunçáo et al. 2001).

Long et al. (2002) have recently described a large biomass Zn hyperaccumulating plant, i.e., Sedum alfredii. Similarly, Wang et al. (2003) have reported on the discovery of two new plants with potential for phytoremediation of Zn-polluted soils, i.e., Polygonum hydropiper and Rumex acetosa. In the same study, the authors indicate that the consumption of rice grown in paddy soils contaminated with Cd, Cr or Zn may pose a serious risk to human health, because from 22 to 24% of the total metal content in the rice biomass was concentrated in the rice grain. Interestingly, Platanus acerifolia growing on heavily contaminated soil accumulated only very low levels of heavy metals, and this mechanism for excluding metal uptake may have value in crop improvement (Wang et al. 2003).

Holcus lanatus L. genotypes tolerant to Zn toxicity seem to grow better than Zn-sensitive genotypes, even in Zn-deficient soil, because of their greater capacity for taking up Zn from Zn-deficient soil, revealing the coexistence of traits for tolerance to Zn toxicity and Zn deficiency in a single plant genotype (Rengel 2000).

Metal responses in the metallophyte Arabidopsis halleri, a close relative to the model plant A. thaliana, that is Cd hypertolerant and Zn hyperaccumulating, have been studied, and metal-regulated genes isolated and molecularly analyzed as interesting candidate genes for phytoremediation (Bert et al. 2000; Dahmani-Muller et al. 2000; Clemens 2001; Macnair 2002). Unlike Thlaspi, A. halleri seems to be largely allogamous, seeds profusely over a longer growing season and can also spread vegetatively by stolons (Baker & Whiting 2002). Macnair (2002) demonstrated that the heritability of Zn accumulation was between 25 and 50%, the highest yet recorded for any hyperaccumulator, probably because of the outbreeding nature of A. halleri. Intriguingly, the genetic variability of Zn accumulation in A. halleri was manifested more when grown in low-Zn media than in high (Baker & Whiting 2002).

Zinc-tolerant callus lines of Brassica spp. have been developed, and this might help in the selection and characterization of heavy metal tolerance in plants for breeding programmes (Rout et al. 1999).

In a paper focusing on soil solution Zn and pH dynamics during phytoextraction using T. caerulescens J. & C. Presl, data indicate that the potential of this hyperaccumulator to remove Zn from contaminated soil may not be related either to acidification of the rhizosphere (McGrath et al. 1997; Luo et al. 2000) or to exudation of specific metal-mobilizing compounds (Zhao et al. 2001). Intriguingly enough, some studies have provided evidence suggesting that roots of T. caerulescens are able to sense and actively forage in the Zn rich patches in soil (Schwartz et al. 1999; Whiting et al. 2000).

A modified glass bead compartment cultivation system for studies on nutrient and trace metal uptake by arbuscular mycorrhiza using two host plant species, maize (Zea mays L.) and red clover (Trifolium pratense L.), and two arbuscular mycorrhizal fungi, Glomus mossae and G. versiforme, found a striking, very high affinity of the fungal mycelium for Zn, suggesting the potential use of arbuscular mycorrhiza in the phytoremediation of Zn-polluted soils (Chen et al. 2001).

2.2. Cadmium

Cadmium is one of the more mobile heavy metals in the soil-plant system, easily taken up by plants and with no essential function known to date (Lehoczky et al. 2000). This element can accumulate in plants without causing toxicity symptoms (Lehoczky et al. 1998).

In soybean plants, results reveal that the content of Cd in different parts of the plants was roots > > stems > seeds, indicating that the accumulation of Cd by roots is much larger than that of any other part of the soybean plant, and might cause deleterious effects to root systems (i.e., decreased nodulation, changes in the ultrastructure of root nodule, etc.) (Chen et al. 2003).

It has been suggested that vetiver grass could be used to remediate Cd-polluted soil, since it accumulated 218 g Cd ha^{-1} at a soil Cd concentration of 0.33 mg Cd kg^{-1} (Chen et al. 2000). Although sequestration of Cd by rhizosphere microorganisms may have an important influence on plant Cd uptake, further research is still required to establish whether the accumulation of Cd by rhizobacteria inhibits, or accelerates, Cd uptake by the host plant (Robinson et al. 2001).

Recent works have aimed to identify the role of antioxidative metabolism in heavy metal tolerance in *T. caerulescens* (Boominathan & Doran 2003a, b). Hairy roots were used to test the effects of high Cd environments, demonstrating that metal-induced oxidative stress occurs in hyperaccumulator tissues even though growth is unaffected by the presence of heavy metals. Superior antioxidant defenses, particularly catalase activity, may play an important role in the hyperaccumulator phenotype of *T. caerulescens*.

Phragmites australis plants were exposed to a high concentration of Cd, finding out that most of this element accumulated in roots, followed by leaves (Iannelli et al. 2002). In roots from Cd-treated plants, both the high amount of glutathione and the parallel increase of glutathione-S-transferase activity seemed to be associated with an induction of the detoxification processes in response to the high Cd concentration. Superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase activities as well as reduced and oxidized glutathione contents in all samples of leaves, roots and stolons were increased in the presence of Cd. Despite the fact that Cd has a redox characteristic not compatible with the Fenton-type chemistry that produces active oxygen species, Cd tolerance in *Phragmites* plants might be associated to the efficiency of these mechanisms.

The effect of Fe status on the uptake of Cd and Zn by two ecotypes of *T. caerulescens*, i.e., Ganges and Prayon (the former being far superior in Cd uptake) was studied (Lombi et al. 2002). Moreover, the *T. caerulescens zip* (Zn-regulated transporter/Fe-regulated transporter-like protein) genes, *TcZNT1-G* and *TcIRT1-G*, were cloned from Ganges and their expression under Fe-sufficient and -deficient conditions analyzed. Cadmium uptake was significantly enhanced by Fe deficiency in Ganges, while Zn uptake was not influenced by the Fe status of the plants in either of the ecotypes. These results are in agreement with the gene expression study, since the abundance of *ZNT1-G* mRNA was always similar, independently of the Fe status or ecotype, while that of *TcIRT1-G* mRNA was greatly increased only in Ganges root tissue under Fe-deficient conditions, suggesting a possible relationship with an up-regulation in the expression of genes encoding Fe uptake, possibly TcIRT1-G (Lombi et al. 2002).

Recently, Song et al. (2003) reported on the utility of the yeast protein YCF1, a protein which detoxifies Cd by transporting it into vacuoles, for the remediation of Cd and Pb contamination, finding out that transgenic *Arabidopsis thaliana* plants overexpressing YCF1 showed enhanced tolerance and accumulated greater amounts of Cd and Pb. Interestingly enough, Lahner et al. (2003) analyzed several essential and nonessential elements in shoots of 6000 mutagenized *A. thaliana* plants, demonstrating the utility of genomic scale profiling of nutrients and trace elements as a functional genomics tools. A better understanding of how plants handle mineral elements has the potential of yielding new phytoremediation capabilities (Rea, 2003).

While Cd detoxification is certainly a complex phenomenon, probably under polygenic control, Cd real tolerance found in mine plants seems to be a simpler phenomenon, possibly involving only monogenic/oligogenic control (Sanità di Toppi & Gabbrielli 1999). These authors concluded that adaptive tolerance is supported by constitutive detoxification mechanisms, which in turn rely on constitutive homeostatic processes.

Symmetric and asymmetric somatic hybridizations have been used to introduce toxic metal-resistant traits from *T. caerulescens* into *Brassica juncea* (Dushenkov et al. 2002). *B. juncea* hypocotyl protoplasts were fused with *T. caerulescens* mesophyll protoplasts, and all putative hybrids had morphological characteristics of *B. juncea*. Hybrid plants, produced by asymmetric somatic hybridization between the two species, demonstrated high metal accumulation potential, tolerance to toxic metals, and good biomass production. In any case, *B. juncea* is by itself a good candidate for efficient phytoextraction of heavy metals -such as Cd- from polluted soils (Schneider et al. 1999).

Two years after the toxic spill caused by the failure of a tailing pond dam at the Aznalcollar pyrite mine (SW Spain) in 1998, none of the trace elements measured -As, Cd, Cu, Pb, Tl- reached levels either phytotoxic or toxic for humans or animals in seeds and the above-ground part of spill-affected sunflower plants (Madejon et al. 2003). However, the potential for phytoextraction of these plants is very low, though they might be useful for soil conservation. The production of oil (usable for industrial purposes, whose production

was greater in the spill-affected plants as compared with unaffected sunflower plants growing in adjacent soil) may add some value to this crop (Madejon et al. 2003).

Field and glasshouse investigations were conducted of the responses on two legumes (field pea and fodder vetch) and three non-leguminous crops (maize, wheat and rapeseed) to the heavy metals Cd, Cr, Zn, Pb, Cu and Mn in soil with multiple metal contamination (Wang et al. 2002a). Among the crops, maize had the highest concentrations of Mn, Zn and Cd, while wheat was the highest accumulator of Pb. More Cd was accumulated in the grain of wheat than of maize, suggesting that growing wheat would represent a higher risk of food contamination than growing maize in Cd-contaminated soil. It was concluded that maize could perhaps be used for phytoremediation of lightly contaminated soils (Wang et al. 2002a).

The tolerance of *T. fragiferum* L. to heavy metals makes it a good prospect for reclamation projects (Jauert et al. 2002). In these plants, there seems to be a negative correlation between rhizosphere pH and Cd uptake.

Mejare & Bulow (2001) reviewed the metal-binding proteins and peptides in bioremediation by microorganisms and phytoremediation of heavy metals, with special emphasis on Cd, indicating that the expression of these molecules to enhance heavy metal accumulation and/or tolerance has great potential.

To augment higher plant metal sequestration, the yeast metallothionein CUP1 (metallothioneins are metal-binding proteins that confer heavy metal tolerance and accumulation) was introduced into tobacco plants, and the cup1 gene expression and Cu and Cd phytoextraction were determined (Thomas et al. 2003). Although pooled leaves of transgenic plants contained two to three times the Cu content as that of the control plants, CUP1 seedling did not significantly sequester or demonstrate tolerance to Cd.

Phytosiderophores, Fe chelators excreted by graminaceous plants under conditions of Fe limitation, also complex Cd. Cadmium has been shown to increase the rate of phytosiderophore 2′-deoxymugineic acid release in maize under both Fe-sufficient and Fe-limiting conditions (Hill et al. 2002). Collectively, results indicated that Cd stress causes Fe deficiency symptoms that result in greater 2′-deoxymugineic acid production by maize roots, and then the 2′-deoxymugineic acid appears to reduce Cd accumulation.

Hairy roots were used to investigate Cd uptake in *T. caerulescens*, finding out that the accumulation of this element increased after treating the roots with H(+)-ATPase inhibitor (Nedelkoska & Doran 2000). Measurement of Cd levels revealed significant differences in the responses of *T. caerulescens* and *Nicotiana tabacum*: most metal was transported directly into the symplasm of N. tabacum roots within 3 days of exposure, while, in contrast, T. caerulescens roots stored virtually all of their Cd in the cell wall fraction for the first 7 to 10 days. This delay in transmembrane uptake may represent an important defensive strategy against Cd poisoning, allowing time for activation of intracellular mechanisms for heavy metal detoxification (Nedelkoska & Doran 2000). In this respect, it should be noted that, due to their fast growth rates and biochemical stability, 'hairy root' cultures remain unsurpassed as the choice for model root systems with a wide range of applications, including as a model for phytoremediation (Shanks & Morgan 1999).

Hairy roots of the Cd hyperaccumulator *T. caerulescens* have been shown to contain high constitutive levels of citric, malic and malonic acids (Boominathan & Doran 2003a, b). *T. caerulescens* hairy roots remained healthy and grew well at high Cd concentrations, with most of the Cd being localized in the cell walls. Growth of *T. caerulescens* hairy roots was severely reduced in the presence of diethylstilbestrol, an inhibitor of plasma membrane H^+-ATPase. Treatment with diethylstilbestrol increased the concentration of Cd in the symplasm of *T. caerulescens* about 6-fold with retention of root viability. These results suggest that the mechanisms of Cd tolerance and hyperaccumulation in *T. caerulescens* hairy roots are capable of withstanding the effects of plasma membrane depolarization (Boominathan & Doran 2003a, b).

Cadmium causes a transient depletion of glutathione and an inhibition of antioxidative enzymes, especially of glutathione reductase (Schutzendubel & Polle 2002). The depletion of glutathione is apparently a critical step in Cd sensitivity, and there are indications that Cd, when not detoxified rapidly enough, may trigger a sequence of reactions leading to growth inhibition, stimulation of secondary metabolism, lignification

and, finally, cell death, in contrast to the idea that Cd results in unspecific necrosis (Schutzendubel & Polle 2002). Mycorrhization stimulates the phenolic defence system in the *Paxillus-Pinus* mycorrhizal symbiosis, but it is still not known whether mycorrhization protects roots from Cd-induced injury by preventing access of Cd to sensitive extra- or intracellular sites, or by excreted or intrinsic metal-chelators, or by any other defence system (Schutzendubel & Polle 2002). Thus, the development of stress-tolerant plant-mycorrhizal associations may be a promising new strategy for phytoremediation.

To better examine the phytoremediation of transgenic Indian mustard (*B. juncea*) plants, overproducing the enzymes gamma-glutamylcysteine synthetase or glutathione synthetase (which have increased levels of the metal-binding thiol peptides phytochelatins and glutathione, and enhanced Cd tolerance and accumulation), and overexpressing adenosine triphosphate sulfurylase (shown to have higher levels of glutathione and total thiols), a greenhouse experiment was conducted in which the transgenics were grown on metal-contaminated soil collected from a USEPA Superfund site (Bennett et al. 2003). All transgenics remove significantly more metal from the soil compared with wild-type Indian mustard, confirming the importance of metal-binding peptides for plant metal accumulation. This study is the first to demonstrate enhanced phytoextraction potential of transgenic plants using polluted environmental soil.

Previously, Indian mustard had been genetically engineered to overexpress the *Escherichia coli gshI* gene encoding gamma-glutamylcysteine synthetase, targeted to the plastids, obtaining seedlings with increased tolerance to Cd and higher concentrations of phytochelatins, gamma-GluCys, glutathione, and total non-protein thiols compared with wild-type seedlings (Zhu et al. 1999b). In the presence of Cd, glutathione synthetase is rate limiting for the biosynthesis of phytochelatins and glutathione (their precursor) (Zhu et al. 1999a).

In order to study the nature of Cd binding in phytochelatins and related cysteine-rich polypeptides in maize (*Z. mays*), Pickering et al. (1999) used X-ray absorption spectroscopy to reveal a predominantly tetrahedral coordination of Cd by sulfur in those molecules, suggesting the presence of a polynuclear Cd cluster in maize phytochelatin.

The effect of $Ca(OH)_2$ addition on immobilization and phytoavailability of Cd from soils was evaluated using *B. juncea* plants (Bolan et al. 2003b). The addition of $Ca(OH)_2$ increases soil pH, thereby increasing the adsorption of Cd, and effectively reducing Cd phytotoxicity. Since there is no direct evidence for $CdCO_3$ or $Cd(OH)_2$ precipitation in the variable charge soil used for the plant growth experiment, alleviation of phytotoxicity is attributed primarily to immobilization of Cd by enhanced pH-induced increases in negative charge.

The *A. thaliana* gene encoding phytochelatin synthase (AtPCS) has been expressed in *E. coli*, observing a marked accumulation of phytochelatins with a concomitant decrease in glutathione cellular content (Sauge-Merle et al. 2003). When the bacterial cells expressing *AtPCS* are placed in the presence of Cd or As, cellular metal contents are significantly increased. This opens up the possibility of using genes from the phytochelatin biosynthetic pathway to design bacteria (bioremediation) or higher plants (phytoremediation) with increased abilities to accumulate toxic metals. By making use of nuclear microscopy techniques, such as micro-PIXE, it has been observed that Cd is sequestered within the trichomes on the leaf surface of *A. thaliana* (Ager et al. 2002).

Using the γ-glutamylcysteine synthetase inhibitor, L-buthionine-[S, R]-sulphoximine (BSO), the role for phytochelatins was evaluated in Cu, Cd, Zn, As, Ni, and Co tolerance in non-metallicolous and metallicolous hypertolerant populations of *Silene vulgaris*, *T. caerulescens*, *H. lanatus* and *Agrostis castelana* (Schat et al. 2002). Phytochelatin-based sequestration seems to be essential for constitutive tolerance but not for hypertolerance to these metals. Cadmium sensitivity is considerably increased by BSO (an inhibitor of phytochelatin synthesis), although exclusively in plants lacking Cd hypertolerance, suggesting that adaptative Cd hypertolerance is not dependent on phytochelatin-mediated sequestration (Schat et al. 2002).

Phytochelatin-based Cd sequestration is generally considered essential for constitutive Cd tolerance in organisms with functional *PCS* genes (Cobbett & Goldsbrough 2002; Schat et al. 2002). The role of PCs in Cd detoxification was

supported by the isolation of two mutants of *Arabidopsis*, *cad1* and *cad2* (deficient in PC and GSH biosynthesis, respectively), which were more sensitive to Cd (Cobbet et al. 1998). Similarly, a strong Cd-induced PC accumulation has been observed in both non-tolerant *Thlaspi* species (*T. arvense*) (Ebbs et al. 2002) and non-tolerant ecotypes of *T. caerulescens* (Schat et al. 2002). Furthermore, Salt et al. (1995b) found enough PCs to chelate all the Cd in the roots of Cd-treated *B. juncea* plants. These authors consistently found the majority of Cd bound to S ligands, with a probable Cd-S$_4$ coordination, and a bond length coincident with that of the purified Cd-PC complex. A strong Cd-induced PC accumulation has been reported in Cd-tolerant *T. caerulescens* ecotypes (i.e., Prayon) (Ebbs et al. 2002) as well as in a Cd-tolerant ecotype isolated from calamine soils (Schat et al. 2002). However, the comparison between non-tolerant and Cd-tolerant ecotypes led to the conclusion that PCs can only play a crucial role for Cd detoxification in non-tolerant ecotypes (Ebbs et al. 2002; Schat et al. 2002). Two main arguments sustain this conclusion: (i) at equal or higher root or shoot Cd internal concentration, PCs accumulation is lower in the more tolerant hyperacumulator ecotype (the more tolerant the ecotype, the lower PC-SH to Cd molar ratio); and (ii) the BSO imposed hypersensitivity to Cd in non-tolerant ecotype was not apparent in the tolerant ecotype, irrespective to the level of Cd exposure. These findings, regarding the lack of a significant role for PCs in *T. caerulescens* Cd hyperaccumulator ecotypes, are consistent with those described for metal tolerant ecotypes of *S. vulgaris* (De Knecht et al. 1994).

Translocation of Cd in the xylem sap appears to be driven by transpiration from the leaves and to be independent of PC production (Salt et al. 1995b). Complexation with low molecular weight organic ligands, such as citrate and histidine, facilitates metal loading into the xylem. Cadmium K-edge EXAFS of xylem sap isolated from B. juncea plants exposed to 0.6 mg Cd ml^{-1} for 7 days showed Cd interactions with N or O, with bond lengths different to that of Cd-PC (Salt et al. 1999). However, very recently, Gong et al. (2003) have found the first evidence of long-distance root-to-shoot transport of PCs and Cd in plants. They observed that the transgenic expression of wheat *TaPCS1* in roots of *cad1-3* mutants of *Arabidopsis*

supressed Cd sensitivity, reduced Cd accumulation in roots, and, most interestingly, led to PC accumulation in rosette leaves and stems. Furthermore, Gong et al. (2003) found a BSO imposed root-to-shoot transport inhibition in *TaPCS1* transgenic *cad1-3 Arabidopsis* mutant. As above mentioned, shoot cellular sequestration through vacuolar compartimentation appears to be a key component in Zn detoxification in *T. caerulescens*, and the same may hold true for Cd detoxification (Ebbs et al. 2002; Schat et al. 2002). Tolerant populations of *T. caerulescens* may sequester Cd in leaf cells more efficiently, either via tonoplast transporters for PC and/or a Cd-PC complex (Ebbs et al. 2002).

A Type 1 metallothionein gene *mcMT1* was cloned from the Cu induced cDNA library of *Festuca rubra* cv. Merlin and by means of functional complementation studies using *Saccharomyces cerevisiae cup1* mutant ABDE-1 (metal sensitive), the functional nature of this *mcMT1* gene in sequestering both essential (Cu, Zn) and non-essential metals (Cd, Pb, Cr) was confirmed (Ma et al. 2003).

It is interesting to note that phytochelatin synthase is not only restricted to plants and some fungi, as was once thought, but also has an essential role in heavy-metal detoxification in the model nematode *Caenorhabditis elegans*, so now phytochelatins, those post-translationally synthesized peptides, will also be investigated from a clinical parasitological standpoint (Vatamaniuk et al. 2002).

Early transcriptional responses of a cell wall-deficient mutant of *Chlamydomonas reinhardtii* to heavy metal stress have been investigated, identifying, sequencing and quantifying the induction of a number of transcripts that are up-regulated by exposure to Cd (Rubinelli et al. 2002). These *Chlamydomonas* strains could be useful for functional genomics studies of metal stress. In addition, the magnitude of induction and functional analyses suggest possible utility for these genes in the study of metal stress sensing in green plants and development of phytoremediation strategies.

Hemp (*Cannabis sativa* L.) has been used to examine its capability as a renewable resource to decontaminate heavy metal polluted soils (Linger et al. 2002). Metal accumulation in different parts of the plant was studied (i.e., seeds, leaves, fibres and hurds), and the highest concentrations of all

examined metals (i.e., Ni, Pb, Cd) are found in the leaves. Hemp shows a phytoremediation potential of 126 g Cd ha^{-1} vegetation per period.

In their studies on the effect of mixed Cd, Cu, Ni, and Zn at different pHs upon alfalfa growth and heavy metal uptake, Peralta-Videa et al. (2002) found that the maximum relative uptakes (element in plant/element in soil-water-solution) are 26 times for Ni, 23 times for Cd, 12 times for Zn, and 6 times for Cu, indicating the ability of alfalfa plants to take up elements from a soil matrix contaminated with a mixture of these metals.

2.3. Lead

Once introduced into the soil matrix, Pb is very difficult to remove. The capacity of the soil to adsorb Pb increases with increasing pH, cation exchange capacity, organic carbon content, soil/water Eh (redox potential) and phosphate levels (United States Environmental Protection Agency 1992).

A model for the uptake, translocation, and accumulation of Pb by maize for the purpose of phytoextraction has been proposed, suggesting that precipitation of Pb as a Pb-phoshate is one of the most important mechanisms in this system, with maximum saturable uptake rate of Pb and effective roots mass being also possible key plant parameters (Brennan & Shelley 1999).

After collecting soil material and plant tissue along transects in two heavily contaminated soil facilities and analyzing them for metal content, it was observed that tissue Pb correlated slightly with exchageable and soluble soil Pb, but tissue Cd was poorly correlated with soil Cd species (Pichtel et al. 2000). Lead and Cd uptake was maximal in *Agrostemma githago* root and in *Taraxacum officinale*, respectively. Those plants that removed most Pb and Cd were predominantly herbaceous species, some of which produce sufficient biomass to be of practical use for phytoextraction. The ability of *T. officinale* and *Ambrosia artemisiifolia* to successfully remove soil Pb and Cd during repeated croppings was demonstrated in growth chamber studies (Pichtel et al. 2000).

Certain plants (mostly, belonging to the *Brassicaceae*, *Euphorbiaceae*, *Asteraceae*, *Lamiaceae*, and *Scrophulariaceae* families) have been identified which have the potential to uptake Pb (United States Environmental Protection Agency

2000a,b). *B. juncea* has been found to have a good ability to transport Pb from roots to the shoots. A study on the effects of different concentrations of Pb on the uptake and accumulation of this element by the roots, hypocotyls and shoots of *B. juncea* var. *megarrhiza* showed that, although *B. juncea* has considerable ability to remove Pb from solutions and accumulate it, the plants transported and concentrated only a small amount of Pb in hypocotyls and shoots (Liu et al. 2000).

T. rotundifolium has been found to grow in soils contaminated with Pb. In any case, one major factor limiting the potential for Pb phytoextraction is low metal bioavailability for plant uptake. To overcome this limitation, synthetic chelators have been proposed to be added to the soil to increase the amount of available Pb (United States Environmental Protection Agency 2000a,b). Sahi et al. (2002) reported on a leguminous fast growing shrub (*Sesbania drummondii*) that accumulates up to 10,000 mg Pb kg^{-1} in shoot after exposure to a Pb-contaminated hydroponic solution. Addition of EDTA (100 μM) to a medium containing 1 g Pb l^{-1} increased uptake by 21%.

A genetically modified *Nicotiana glauca* R. Graham (shrub tobacco) has proven to accumulate Pb, making it especially promising for phytoremediation (Gisbert et al. 2003). This plant has a wide geographic distribution, is fast-growing with a high biomass, and is repulsive to hervibores. The induction and overexpression of a wheat gene encoding phytochelating synthase in this plant greatly increased its tolerance to Pb and Cd, and seedlings of the transformed plants accumulated double concentration of Pb compared to the wild type.

Using radiolabeled recombinant calmodulin as a probe to screen a tobacco cDNA library, Arazi et al. (1999) discovered a protein, NtCBP4, that can modulate plant tolerance to heavy metals. Several independent transgenic lines expressing NtCBP4 had higher than normal levels of NtCBP4, exhibiting improved tolerance to Ni and hypersentitivity to Pb, which are associated with reduced Ni accumulation and enhanced Pb accumulation, respectively. This was the first report of a plant protein (probably involved in metal uptake across the plasma membrane) that modulates plant tolerance and accumulation of Pb. This gene could be useful for improving phytoremediation strategies.

2.4. *Arsenic*

As pointed out in the review by Oremland and Stolz (2003), As pollution is a problem of critical importance currently affecting the health of millions of people worlwide. In the above mentioned paper, the authors reviewed what is known about As-metabolizing bacteria and indicated that arsenite oxidation is being studied as the basis for bioremediation of systems where arsenite, As(III), is a pollutant.

In this context, phytoremediation has recently been proposed as an effective tool in As cleanup. In fact, the Chinese brake fern (*Pteris vittata*) has been reported to hyperaccumulate As to extremely high concentrations, up to 23,000 μg arsenic g^{-1}, in its shoots (fronds) (Ma et al. 2001). This primitive plant actually thrives on As, doubling its biomass in one week when subjected to 100 ppm As. The striking difference between *P. vittata* and As non-accumulators is the remarkable transport of this element from roots to shoots in *P. vittata*, accumulating up to 95% of the As in the above-ground tissue (Doucleff & Terry 2002). Zhang et al. (2002) have hypothesized that the brake fern takes up As as arsenate, subsequently converting it to arsenite within the plant. *P. vittata* has been shown to reach a bioconcentration factor (ratio of plant As concentration to water-soluble As in soil) of 1450 (Tu et al. 2002). Once the molecular mechanisms involved in As uptake and transport by *P. vittata* are known, genes responsible for the remarkable abilities of this plant could be used to transform fast-growing, high-biomass phytoremediators (Doucleff & Terry 2002). Other plants such as *Pityrogramma calomelanos*, *Mimosa pudica*, and *Melastoma malabrathricum* might be suitable for As phytoremediation (Visoottiviseth et al. 2002). *P. calomelanos* accumulates most of this metalloid in the fronds while the rhizoids contain the lowest concentrations of As (Francesconi et al. 2002).

A transgenic system for removing As from soil, inserting two genes, *arsC* (arsenate reductase) and *ECS* (γ-glutamylcysteine synthetase) from *E. coli* into *A. thaliana* plants has been developed (Dhankher et al. 2002). When grown on As, the transgenic plants accumulated 4- to 17-fold greater fresh shoot weight and accumulated 2- to 3-fold more As per gram of tissue than wild type or plants expressing γ-*ECS* or *ArsC* alone (Dhankher et al. 2002). In any case, the tolerance and accumulation values reached by these transgenic plants were very distant to those of *P. vittata*.

Sauge-Merle et al. (2003) have described a different approach, expressing the *A. thaliana* gene encoding phytochelatin synthetase (AtPCS) in *E. coli*. When bacterial cells expressing *AtPCS* were placed in the presence of As, cellular metal contents were increased 50-fold. More research is still needed on the possibility of using genes of the phytochelatin biosynthetic pathway to design bacterial strains or higher plants with increased abilities to acumulate As for remediation processes.

In *B. juncea*, once in the shoot, As is stored as an As(III)tris-thiolate complex, being the most probable thiolate donors either glutathione or phytochelatins (Pickering et al. 2000). In this respect, the addition of the dithiol As chelator dimercaptosuccinate to As-contaminated soils has been suggested to promote As bioaccumulation in plant shoots.

A study of the influence of root temperature on phytoaccumulation of As, among other elements, in potato plants (*Solanum tuberosum* L. var. Spunta), induced by the application of mulches, indicated that different root temperatures were accompanied by significantly different responses in As phytoaccumulation (Baghour et al. 2001). In terms of the relative distribution of the phytoaccumulated metals (with respect to the total of the plant), As accumulated mainly in the roots and leaflets. With regard to phytoremediation using tomato plants, it is important to consider the thermal regime of the soil to optimize phytoextraction (Baghour et al. 2001).

The γ-glutamylcysteine synthetase inhibitor, L-buthionine-[S, R]-sulphoximine (BSO), dramatically increases As sensitivity, both in non-adapted and As-hypertolerant plants, showing that phytochelatin-based sequestration is essential for both normal constitutive tolerance and adaptative hypertolerance to this metalloid (Schat et al. 2002).

3. General new findings and outlook

Improvement of plants by genetic engineering opens up new possibilities for phytoremediation (Kärenlampi et al. 2000). Also, genes involved in the regulation of the partitioning of metals

between root and shoot will be of high interest in the future (Delhaize 1996).

So far, only a few cases have been reported where metal uptake, transport and accumulation as well as metal tolerance have been succesfully altered. These include Hg ion reduction causing improved resistance and phytoextraction (Heaton et al. 1998; Rugh et al. 1996, 1998a,b), and metallothionein causing enhanced Cd tolerance (Misra & Gedamu 1989; Evans et al. 1992; Elmayan & Tepfer 1994; Hattori et al. 1994; Hasegawa et al. 1997; Kärenlampi et al. 2000; Krämer & Chardonnens 2001).

Very exciting works on the application of molecular genetic approaches to improve metal phytoremediation were published by Meagher's team in relation to Hg pollution (Rugh et al. 1996, 1998a,b). They used expression of *merA* gene (mercuric reductase, MerA, converts toxic Hg^{2+} to the less toxic, relatively inert Hg^0 in bacteria) in transgenic plants for the remediation of Hg pollution. A mutagenized *merA* sequence, merApe9, was constructed and transgenic *A. thaliana* seedlings expressing this sequence germinated and grew on Hg-containing medium, evolving considerable amounts of Hg^0 relative to control plants. The ability of yellow poplar (*Liriodendron tulipifera*) tissue cultures and plantlets to express modified mercury reductase gene constructs has also been reported (Rugh et al. 1998a). The significance of the study of Meagher's research group is that it can lead to the more efficient and affordable cleanup of environmental Hg pollution, and in a broader context, it proves the power of genetic engineering for phytoremediation (Pilon-Smits & Pilon 2000). Most recently, Bizily et al. (2003) have engineered plants that express the bacterial Hg resistance enzymes organomercurial lyase MerB and mercuric ion reductase MerA. The *merB* gene was engineered to target MerB for accumulation in the endoplasmic reticulum and for secretion to the cell wall. MerB protein in endoplasmic reticulum-targeted plants appeared to accumulate in large vesicular structures and moderate increases in targeted MerB expression led to significant gains in detoxification.

Hyperaccumulators such as *T. caerulescens* or *B. juncea* should be good sources for genes suitable for phytoremediation. Since tolerance and accumulation are largely independent properties, they should be both engineered to get a suitable plant for metal phytoextraction (Macnair et al. 1999; Käremlampi et al. 2000). With respect to metal phytoextraction, two strategies could be considered: (i) convert slow-growing, low-biomass hyperaccumulators into fast-growing, high-biomass varieties, or, alternatively, (ii) introduce metal-hyperaccumulation traits into fast-growing, high-biomass plants (Cunningham & Ow 1996).

Raskin (1996) suggested that transgenic plants could be developed to secrete metal-selective ligands into the rhizosphere which could specifically solubilize elements of phytoremediation interest.

Since little is known about the rhizosphere of hyperaccumulators, Delorme et al. (2001) compared the influence of the well-known *T. caerulescens* with the effects of *T. pratense* on soil microbes, finding out that microbial populations were higher in soils planted with *T. pratense* but higher ratios of metal-resistant bacteria were found in the presence of *T. caerulescens*. The authors hypothesized that *T. caerulescens* acidifies its rhizosphere, increasing available metals around the roots and, consequently, increases selection for metal-resistant bacteria.

Since metal transport from the cytosol to the vacuole is thought to be an important component of ion tolerance and of a plant's potential or use in phytoremediation, some studies have been performed to understand this process (Hirschi et al. 2000). In fact, in tobacco (*N. tabacum*) plants, it has been found that the *Arabidopsis* antiporter CAX2 (calcium exchanger 2) may be a key mediator of the metal transport from the cytosol to the vacuole (Hirschi et al. 2000). Tobacco plants expressing CAX2 accumulated more Ca, Cd, and Mn and were more tolerant to elevated Mn levels. Expression of CAX2 also increased Cd and Mn transport in isolated root tonoplast vesicles. Modulation of this transporter could be useful for phytoremediation.

Some authors (Krämer & Chardonnens 2001) consider that field trials have suggested that the rate of contaminant removal using conventional traits and growth conditions is insufficient, and so the introduction of novel traits into high biomass plants in a transgenic approach is needed for effective phytoremediation. In any case, the engineering of a phytoremediator plant requires the optimization of processes such as trace element mobilization in the soil, uptake into the

root, and detoxification and allocation within the plant (Krämer & Chardonnens 2001; Singh et al. 2003).

Molecular techniques such as the analysis of molecular variance of the random amplified polymorphic DNA markers are useful to investigate the genetic diversity and heavy metal tolerance in plant populations, providing the opportunity to investigate the first steps in the differentiation of plant populations under severe selection pressure and to select plants for phytoremediation (Mengoni et al. 2000).

One strategy for increasing the efficiency of phytoextraction is to increase metal translocation to the shoot by increasing plant transpiration. To determine whether genetically increased transpiration would increase the efficiency of phytoextraction, seeds of *B. juncea* were mutagenized with ethyl methanesulfonate, and mature plants were then self-pollinated (Gleba et al. 1999). In subsequent steps, a line in which the transpiration rate exceeded that of the wild-type plants by 130% in soil, phytoextraction of Pb was tested in Pb-contaminated soil amended with EDTA. The high-transpiration line phytoextracted 104% more Pb than the wild-type *B. juncea*.

A much better understanding of the genetic, biochemical and physiological basis of metal hyperaccumulation in plants is of key importance for the success of phytoremediation. The mechanisms deployed in the acquisition of essential heavy metal micronutrients have not been clearly defined although a number of genes have now been identified which encode potential transporters. Three classes of membrane transporters have been implicated in the transport of heavy metals in a variety of organisms and could serve such a role in plants: (i) the heavy metal (CPx-type) ATPases, (ii) the natural resistance-associated macrophage protein (Nramp) family, and (iii) members of the cation diffusion facilitator (CDF) family (Williams et al. 2000), recently renamed as cation efflux family (CEF) (Maser et al. 2001).

Members of the ZIP gene family are also capable of transporting a variety of cations, including Cd, Fe, Mn and Zn (Guerinot 2000). Information on where in the plant each of the ZIP transporters functions and about its regulation, would open the possibility of manipulating plant mineral status with an eye to developing crops that bioaccumulate or exclude toxic metals.

According to Rugh et al. (1998a), the exceptional physiological abilities of plants could be augmented with genes from the "molecular tool-boxes" of microbial metabolism to allow the development of powerful weapons for pollution control.

In recent years, several key steps have been identified at the molecular level, enabling us to initiate transgenic approaches to engineer the transition metal content of plants (Clemens et al. 2002). In this respect, transgenic tomato plants (*Lycopersicon esculentum* cv. Heinz 902) expressing the bacterial gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase were compared to non-transgenic tomato plants in their ability to grow in the presence of and accumulate Cd, Co, Cu, Mg, Ni, Pb or Zn (Grichko et al. 2000). In general, transgenic tomato plants expressing ACC deaminase acquire a greater amount of metal within the plant tissues, and are less subject to the deleterious effects of the metals on plant growth than the non-transgenic plants.

Finally, and since considerable expectations are placed on genetic modification to produce idealized plants for phytoextraction, it is important to remember that the ever-increasing public concern over the release of genetically modified organisms could force regulators to veto their use. As exposed by Baker & Whiting (2002), it would be prudent to complement genetical approaches with searches for natural hyperaccumulators and further explore conventional plant breeding practices (Chaney et al. 2000), with the additional benefit of cataloging and conserving the unique global biodiversity in mineralized environments. Furthermore, Kramer (2001) suggested that the Ganges *T. caerulescens* ecotype (Lombi et al. 2000) should be considered a model population of to be centrally propagated, crossed and made available for all scientists, similar to seed stocks of *A. thaliana* mutants and ecotypes. This would eventually reduce the genetic variability within the seed population, a major drawback in the work with metal hyperaccumulators.

Although phytoremediation is still a new technology, in the last few years a lot of basic research has been carried out in an attempt to understand how plants take up large quantities of metals, together with the mechanisms of metal translocation from roots to shoots, storage and detoxification. Nevertheless, it is not yet clear how this informa-

tion should be used to efficiently remove metals from polluted fields. But, apart from deeper basic research, more applied projects in the field are needed to clarify the real potential of this technology.

Indeed, before attempting field implementation of this technology, it is essential to conduct basic laboratory work first, if we are to avoid inflated expectations coming from exaggerated claims of success (as was the case with bacterial remediation regarding its contaminant removal efficiency) (Boyajian & Carreira, 1997).

In this respect, two strategies will definitely be considered in the future: (i) optimization of agronomic practices, including fertilization, crop protection chemicals, methods of harvesting and sowing, etc., especially for hyperaccumulating plants, since the knowledge needed for proper cultivation of many of the reported hyperaccumulators is still lacking; and (ii) genetic manipulation of plants, although, as above-mentioned, the utilization of transgenic plants presently does not enjoy high public acceptance, at least regarding the utilization of GM crops for human consumption. However, although certainly extremely difficult to predict, public opinion might be different when transgenic plants are aimed towards environmental cleanup, such as transgenic phytoremediating plants. In any case, before GM techniques can be applied successfully, a detailed knowledge of the mechanisms of uptake, translocation, sequestration, etc. of metals in plants is required, and that information is at present certainly incomplete. After all, the functions of many plant transporters that are central to phytoremediation remain still uncharacterized.

It is likely that conventional breeding techniques, which undoubtedly enjoy high public acceptance, might provide a suitable alternative although, so far, crossing between hyperaccumulators and crops plants has not been successful mainly due to incompatibility problems. Within a species, the use of the selection of individuals with greater metal removal efficiency has apparently not been achieved yet (McGrath et al. 2002). Brewer et al. (1999) used somatic hybridization to combine hyperaccumulating traits with those of higher biomass crop species, but combining genomes and selecting a progeny using non-GM methods requires relatively long periods of time.

One frequently ignored aspect when discussing future directions within the phytoextraction field is the need to develop economically feasible techniques for the disposal of metal-enriched plants or, when practical, for metal recovery. An economical method of reclaiming metals from plant residues is required to eliminate the need for costly off-site disposal. Most importantly, research must also inevitably be focused towards finding ways to avoid transfer of metals to other media, the environment in general, and particularly to the food chain.

Although phytoextraction is not a magic solution, commercially, it is gaining appeal because it is cheaper than conventional clean-up methods. But it is not an easy technology just consisting of picking up some hyperaccumulating plants and placing them in the metal polluted area. On the contrary, it is highly technical, requiring expert project designers with plenty of field experience that carefully choose the proper species and cultivars for particular metals (and combinations of them) and regions, and manage the entire system to maximize pollutant removal efficiency.

For the time being, phytoextraction is not the appropriate choice for all metal polluted sites since, for instance, in many cases, the high metal concentrations and/or the presence of harsh conditions (hostile climate, soil properties) do not allow plant growth. Consequently, phytoextraction will most likely be used in areas with medium to low levels of metals, or as a final polishing step (to reduce the cost and impact of other methods) after other clean-up techniques have been used to treat the hot spots. Inevitably, phytoextraction (limited to the rooting zone) will find its widest application in the remediation of surface-polluted soils.

In any event, phytoextraction of metals definitely holds great potential for the removal of these pollutants from soil. Phytoextraction is a more cost-effective alternative than conventional remediation methods and since many decisions are based on the basis of strict cost-benefit analysis, economic pressures together with public acceptance will probably continue to influence decisions towards these biological methods. Besides, although remediation does not represent profit to the polluting companies, it reduces the probability or magnitude of legal liability, often making it worth the investment.

Phytoextraction is little by little carving out its own niche as a sure and aesthetically pleasing method to remove metals from polluted soils. Although studied extensively in research and in small-scale demonstrations, yet full-scale applications of phytoextraction are currently limited to a small number of projects. Phytoextraction needs a transdisciplinary (not simply multidisciplinary) approach with inputs from many fields such as botany, plant physiology, biochemistry, geochemistry, agricultural engineering, agronomy, soil science, genetic engineering and so on.

Although only the future will tell us whether phytoremediation will become a widely used technology, the study of the utilization of the remarkable ability of plants to remove pollutants from the environment is at present a fascinating field of research.

References

Ager FJ, Ynsa MD, Domínguez-Solís JR, Gotor C, Respaldiza MA & Romero LC (2002) Cadmium localization and quantification in the plant *A. thaliana* using micro-PIXE. Nuclear Instr. Methods in Phy. Res. Section B. Beam Interactions with Mat. and Atoms 189: 494–498

Alkorta I & Garbisu C (2001) Phytoremediation of organic contaminants. Bioresource Technol. 79: 273–276

Arazi T, Sunkar R, Kaplan B & Fromm H (1999) A tobacco plasma membrane calmodulin-binding transporter confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity in transgenic plants. Plant J. 20: 171–182

Assunçáo AGL, Martins PD, De Folter S, Vooijs R, Schat H & Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. 24: 217–226

Baghour M, Moreno DA, Hernández J, Castilla N & Romero L (2001) Influence of root temperature on phytoaccumulation of As, Ag, Cr, and Sb in potato plants (*Solanum tuberosum* L. var. Spunta). J. Environ. Sci. Health Part A Tox. Hazard Subst. Environ. Eng. 36: 1389–1401

Baker AJM (1981) Accumulators and excluders—Strategies in the response of plants toheavy metals. J. Plant Nutr. 3: 643–654

Baker AJM & Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. Biorecovery 1: 81–126

Baker AJM & Whiting SN (2002) In search of the Holy Grail—a further step in understanding metal hyperaccumulation? New Phytol. 155: 1–7

Baker AJM, McGrath SP, Reeves RD & Smith JAC (2000) Metal hyperaccumulator plants: A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry N, Bañuelos G & Vangronsveld J (Eds), Phytoremediation of contaminated soil and water (pp 85–107). Lewis Publisher, Boca Raton, FL, USA

Baker AJM, McGrath SP, Sidoli CMD & Reeves RD (1994a) The possibility of *in situ* heavy metal decontamination of polluted soils using crops of metal-accumulating plants. Resour. Conser. Recycl. 11: 41–49

Baker AJM, Reeves RD & Hajar ASM (1994b) Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). New Phytol. 127: 61–68

Bennet LE, Burkhead JL, Hale KL, Terry N, Pilon M & Pilon-Smits EA (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. J. Environ. Qual. 32: 432–440

Bert V, Macnair MR, de Laguerie P, Saumitou-Laprade P & Petit D (2000) Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). New Phytol. 146: 225–233

Bizily SP, Kim T, Kandasamy MK & Meagher RB (2003) Subcellular targeting of methylmercury lyase enhances its specific activity for organic mercury detoxification in plants. Plant Physiol. 131: 463–471

Bolan NS, Adriano DC, Mani PA & Duraisamy A (2003a) Immobilization and phytoavailability of cadmium in variable charge soils. II. Effect of lime addition. Plant Soil 251: 187–198

Bolan NS, Adriano DC & Naidu R (2003b) Role of phosphorus in (im)mobilization and bioavailability of heavy metals in the soil-plant system. Rev. Environ. Contam. Toxicol. 177: 1–44

Boominathan R & Doran PM (2003a) Organic acid complexation, heavy metal distribution and the effects of ATPase inhibition in hairy roots of hyperaccumulator plant species. J. Biotechnol. 101: 131–146

Boominathan R & Doran PM (2003b) Cadmium tolerance and antioxidative defenses in hairy roots of the cadmium hyperaccumulator, *Thlaspi caerulescens*. Biotechnol. Bioengineer. 20: 158–167

Boyajian GE & Carreira LH (1997) Phytoremediation: a clean transition from laboratory to marketplace. Nat. Biotechnol. 15: 127–128

Brennan MA & Shelley ML (1999) A model of the uptake, translocation, and accumulation of lead (Pb) by maize for the purpose of phytoextraction. Ecol. Engineer. 12: 271–297

Brewer EP, Saunders JA, Angle JS, Chaney RL & McIntosh MS (1999) Somatic hybridization between the zinc accumulator *Thlaspi caer*ulescens and *Brassica napous*. Theore. Appl. Gen. 9: 761–771

Briat JF & Lebrun M (1999) Plant responses to metal toxicity. Comptes Rendus de l′Académie des Sciences - Series III - Sciences de la Vie 322: 43–54

Brooks RR (1988) (Ed) Plants that Hyperaccumulate Heavy Metals. CAB International, Oxon, UK, 356 pp

Brooks RR, Lee J, Reeves RD & Jaffré T (1977) Detection of nickeliferous rocks by analysis of herbarium species of indicator plants. J. Geochem. Explor. 7: 49–57

Brown SL, Chaney RL, Angle JS & Baker AJ (1995) Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci. Soc. Am. J. 59: 125–133

Chaney RL, Li YM, Brown SL, Homer FA, Malik M, Angle JS, Baker AJM, Reeves RD & Chin M (2000) Improving metal hyperaccumulator wild plant to commercial phytoextraction systems: Approaches and progress. In: Terry N, Bañuelos G & Vangronsveld J (Eds), Phytoremediation of contaminated soil and water (pp 129–158). Lewis Publisher, Boca Raton, FL, USA

Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS & Baker AJ (1997) Phytoremediation of metals. Curr. Opin. Biotechnol. 8: 279–284

Chaudhry TM, Hayes WJ, Khan AG & Khoo CS (1998) Phytoremediation—Focusing on accumulator plants that remediate metal-contaminated soils. Australasian J. Ecotoxicol. 4: 37–51

Chen B, Christie P & Li X (2001) A modified glass bead compartment cultivation system for studies on nutrient and trace metal uptake by arbuscular mycorrhiza. Chemosphere 42: 185–192

Chen HM, Zheng CR, Tu C & Shen ZG (2000) Chemical methods and phytoremediation of soil contaminated with heavy metals. Chemosphere 41: 229–234

Chen YX, He YF, Yang Y, Yu YL, Zheng SJ, Tian GM, Luo YM & Wong MH (2003) Effect of cadmium on nodulation and N_2-fixation of soybean in contaminated soils. Chemosphere 50: 781–787

Clemens S (2001) Developing tools for phytoremediation: towards a molecular understanding of plant metal tolerance and accumulation. Int. J. Occup. Med. Environ. Health 14: 235–239

Clemens S, Palmgren MG & Krämer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci. 7: 309–315

Cobbett C, May MJ, Howden R & Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamilcysteine synthetase. Plant J. 16: 73–78

Cobbett C & Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 53: 159–182

Collins YE & Stotzky G (1989) Factors affecting the toxicity of heavy metals to microbes. In: Beveridge TJ & Doyle RJ (Eds), Metal Ions and Bacteria (pp 31–90). Wiley, Toronto, Canada

Cunningham SD & Ow DW (1996) Promises and prospects of phytoremediation. Plant Physiol. 110: 715–719

Dahmani-Muller H, van Oort F, Gélie B & Balabane M (2000) Strategies of heavy metal uptake by three plant species growing near a metal smelter. Environ. Pollut. 109: 231–238

Dahmani-Muller H, van Oort F & Balabane M (2001) Metal extraction by *Arabidopsis halleri* grown on an unpolluted soil amended with various metal-bearing solids: a pot experiment. Environ. Pollut. 114: 77–84

De Knecht JA, van Dillen M, Koevoets PLM, Schat H, Verkleij JAC & Ernst WHO (1994) Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. Plant Physiol. 104: 255–261

Delhaize E. A (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. Plant Physiol. 111: 849–855

Delorme TA, Gagliardi JV, Angle JS & Chaney RL (2001) Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations. Can. J. Microbiol. 47: 773–776

Dhankher OP, Li Y, Rosen BP, Shi J, Salt D, Senecoff JF, Sashti NA & Meagher RB (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and γ-glutamylcysteine synthetase expression. Nat. Biotechnol. 20: 1140–1145

Diels N, van der Lelie D & Bastiaens L (2002) New developments in treatment of heavy metal contaminated soils. Re/Views in Environ. Sci. & Bio/Technol. 1: 75–82

Doucleff M & Terry N (2002) Pumping out the arsenic. Nat. Biotechnol. 20: 1094–1095

Dushenkov S, Skarzhinskaya M, Glimelius K, Gleba D & Raskin I (2002) Bioengineering of a phytoremediation plant by means of somatic hybridization. Int. J. Phytoremediation 4: 117–126

Ebbs S, Lau I, Ahner B & Kochian L (2002) Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214: 635–640

Elmayan T & Tepfer M (1994) Synthesis of a bifunctional metallothionein/b-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. Plant J. 6: 433–440

Entry JA, Rygiewicz PT, Watrud LS & Donnelly PK (2002) Influence of adverse soil conditions on the formation and function of arbuscular mycorrhizas. Adv. Environ. Res. 7: 123–138

Evans KM, Gatehouse JA, Lindsay WP, Shi J, Tommey AM & Robinson NJ (1992) Expression of the pea metallothionein-like gene *PsMTA* in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: Implications for PsMTA function. Plant Mol. Biol. 20: 1019–1028

Evans LD (2002) The dirt on phytoremediation. J. Soil & Water Conservation 57: 12A–15A

Francesconi K, Visoottiviseth P, Sridokchan W & Goessler W (2002) Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: a potential phytoremediator of arsenic-contaminated soils. Sci. Total Environ. 284: 27–35

Gabbrielli R, Pandolfini T, Vergnano O & Palandri MR (1990) Comparison of two serpentine species with different nickel tolerance strategies. Plant Soil. 122: 271–277

Garbisu C & Alkorta I (1997) Bioremediation: principles and future. J. Clean Technol. Environ. Toxic. & Occup. Med. 6: 351–366

Garbisu C & Alkorta I (2001) Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. Bioresource Technol. 77: 229–236

Garbisu C & Alkorta I (2003) Basic concepts on heavy metal soil bioremediation. Eur. J. Min. Proc. & Environ. Protect. 3: 58–66

Garbisu C, Hernández-Allica J, Barrutia O, Alkorta I & Becerril JM (2002) Phytoremediation: A technology using green plants to remove contaminants from polluted areas. Rev. Environ. Health 17: 75–90

Gisbert C, Ros R, de Haro A, Walker DJ, Bernal MP, Serrano R & Navarro-Avino J (2003) A plant genetically modified that accumulates Pb is especially promising for phytoremediation. Biochem. Biophys. Res. Commun. 303: 440–445

Gleba D, Borisjuk NV, Borisjuk LG, Kneer R, Poulev A, Skarzhinskaya M, Dushenkov S, Logendra S, Gleba YY & Raskin I (1999) Use of plant roots for phytoremediation and molecular farming. Proc. Natl. Acad. Sci. USA 96: 5973–5977

Gong JM, Lee DA & Schroeder JI (2003) Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. 2003 Proc. Natl. Acad. Sci. USA 100: 10118–10123

Grichko VP, Filby B & Glick BR (2000) Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. J. Biotechnol. 81: 45–53

Guerinot ML (2000) The ZIP family of metal transporters. Biochim. Biophys. Acta (BBA)-Biomembranes 1465: 190–198

88

Hasegawa I, Terada E, Sunairi M, Wakita H, Shinmachi F & Noguchi A (1997) Genetic improvement of heavy metal tolerance in plants by transfer of the yeast metallothionein gene (CUP1). Plant Soil 196: 277–281

Hattori J, Labbe H & Miki BL (1994) Construction and expression of a metallothionein-beta-glucuronidase gene fusion. Genome 37: 508–512

Heaton ACP, Rugh CL, Wang N & Meagher RB (1998) Phytoremediation of mercury- and methylmercury-polluted soils using genetically engineered plants. J. Soil Contam. 7: 497–509

Hill KA, Lion LW & Ahner BA (2002) Reduced Cd accumulation in Zea mays: a protective role for phytosiderophores? Environ. Sci. Technol. 36: 5363–5368

Hirschi KD, Korenkov VD, Wilganowski NL & Wagner GJ (2000) Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol. 124: 125–133

Homer FA, Morrison RS, Brooks RR, Clement J & Reeves RD (1991) Comparative studies of nickel, cobalt, and copper uptake by some nickel hyperaccumulators of the genus Alyssum. Plant Soil. 138: 195–205

Iannelli MA, Pietrini F, Fiore L, Petrilli L & Massacci A (2002) Antioxidant response to cadmium in Phragmites australis plants. Plant Physiol. Biochem. 40: 977–982

Jauert P, Schumacher TE, Boe A & Reese RN (2002) Rhizosphere acidification and cadmium uptake by strawberry clover. J. Environ. Qual. 31: 627–633

Kamnev AA & van der Lelie D (2000) Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Biosci. Rep. 20: 239–258

Kärenlampi S, Schat H, Vangronsveld J, Verkleij JAC, van der Lelie D, Mergeay M & Tervahauta AI (2000) Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ. Pollut. 107: 225–231

Koppolu L & Clements LD (2003) Pyrolysis as a technique for separating heavy metals from hyperaccumulators. Part I: Preparation of synthetic hyperaccumulator biomass. Biomass & Bioenergy 24: 69–79

Krämer U & Chardonnens AN (2001) The use of transgenic plants in the bioremediation of soils contaminated with trace elements. Appl. Microbiol. Biotechnol. 55: 661–672

Krämer U (2000) Cadmium for all meals—plants with an unusual appetite. New Phytol. 145: 1–5

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM & Smith JAC (1996) Free histidine as a metal chelator in plants that hyperaccumulate nickel. Nature 379: 635–638

Lahner B, Gong J, Mahmoudian M, Smith EL, Abid KB, Rogers EE, Guerinot ML, Harper JF, Ward JM, McIntyre L, Schroeder JI & Salt DE (2003) Genomic scale profiling of nutrient and trace elements in Arabidopsis thaliana. Nat. Biotechnol. 21: 1215–1221

Lasat MM, Baker AJM & Kochian LV (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanism involved in Zn hyperaccumulation in Thlaspi caerulescens. Plant Physiol. 118: 875–883

Lasat MM (2002) Phytoextraction of toxic metals: a review of biological mechanisms. J. Environ. Qual. 31: 109–120

Lehoczky E, Marth P, Szabados I, Palkovics M & Lukács P (2000) Influence of soil factors on the accumulation of cadmium by lettuce. Commun. Soil Sci. Plant Anal. 31: 2425–2431

Lehoczky E, Szabó L & Horváth S (1998) Cadmium uptake by plants in different soils. Commun. Soil Sci. Plant Anal. 29: 1903–1912

Linger P, Müssig J, Fischer H & Kobert J (2002) Industrial hemp (Cannabis sativa L.) growing on heavy metal contaminated soil: fibre quality and phytoremediation potential. Industr. Crops Protect. 16: 33–42

Liu D, Jiang W, Liu C, Xin C & Hou W (2000) Uptake and accumulation of lead by roots, hypocotyls and shoots of Indian mustard (Brassica juncea L.). Bioresource Technol. 71: 273–277

Lombi E, Tearall KL, Howarth JR, Zhao FJ, Hawkesford MJ & McGrath SP (2002) Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator Thlaspi caerulescens. Plant Physiol. 128: 1359–1367

Lombi E, Zhao FJ, Dunham SJ & McGrath SP (2000) Cadmium accumulation in populations of Thlaspi caerulescens and Thlaspi geosingense. New Phytol. 145: 11–20

Long XX, Yang XE, Ye ZQ, Ni WZ & Shi WY (2002) Differences of uptake and accumulation of zinc in four species of Sedum. Acta Botanica Sinica 44: 152–157

Luo YM, Christie P & Baker AJM (2000) Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of Thlaspi caerulescens grown in a Zn/Cd-contaminated soil. Chemosphere 41: 161–164

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y & Kennelley ED (2001) A fern that hyperaccumulates arsenic. Nature 409: 579

Ma M, Lau PS, Jia YT, Tsang WK, Lam SKS, Tam NFY & Wong YS (2003) The isolation and characterization of Type 1 metallothionein (MT) cDNA from a heavy-metal-tolerant plant, Festuca rubra cv. Merlin. Plant Sci. 164: 51–60

Macnair MR (2002) Within- and between-population genetic variation for Zn accumulation in Arabidopsis halleri. New Phytol. 155: 59–66

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P & Petit D (1999) Zinc tolerance and hyperaccumulation are genetically independent characters. Proc. Royal Soc. London B 266: 2175–2179

Madejon P, Murillo JM, Maranon T, Cabrera F & Soriano MA (2003) Trace element and nutrient accumulation in sunflower plants two years after the Aznalcollar mine spill. Sci.Total Environ. 20: 239–257

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA & Guerinot ML (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol. 126:1646–1667

McGrath SP, Shen ZG & Zhao FJ (1997) Heavy metal uptake and chemical changes in the rhizosphere of Thlaspi caerulescens and Thlaspi ochroleucum grown in contaminated soils. Plant Soil 188: 153–159

McGrath SP, Lombi E & Zhao FJ (2001) What's new about cadmium hyperaccumulation? New Phytol. 149: 2–3

McGrath SP, Zhao FJ & Lombi E (2002) Phytoremediation of metals, metalloids, and radionuclides. Adv. Agronomy 75: 1–56

McGrath SP & Zhao FJ (2003) Phytoextraction of metals and metalloids. Curr. Opin. Biotechnol. 14: 277–282

McIntyre T (2003) Phytoremediation of heavy metals from soils. Adv. Biochem. Eng. Biotechnol. 78: 97–123

Meagher RB (2000) Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. 3: 153–162

Mejare M & Bulow L (2001) Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol. 19: 67–73

Mengoni A, Gonnelli C, Galardi F, Gabbrielli R & Bazzicalupo M (2000) Genetic diversity and heavy metal tolerance in populations of *Silene paradoxa* L. (*Caryophyllaceae*): a random amplified polymorphic DNA analysis. Mol. Ecol. 9: 1319–1324

Misra S & Gedamu L (1989) Heavy metal tolerance transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. Theor. Appl. Gen. 78: 161–168

Morgan AJ, Evans M, Winters C, Gane M & Davies MS (2002) Assaying the effects of chemical ameliorants with earthworms and plants exposed to a heavily polluted metalliferous soil. Eur. J. Soil Biol. 38: 323–327

Navari-Izzo F & Quartacci MF (2001) Phytoremediation of metals. Tolerance mechanisms against oxidative stress. Minerva Biotec. 13: 73–83

Nedelkoska TV & Doran PM (2000) Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. Biotechnol. Bioeng. 67: 607–615

O'Connor CS, Leppi NW, Edwards R & Sunderland G (2003) The combined use of electrokinetic remediation and phytoremediation to decontaminate metal-polluted soils: a laboratory-scale feasibility study. Environ. Monit. Assess. 84: 141–158

Oremland RS & Stolz JF (2003) The ecology of arsenic. Science 300: 939–944

Pawlowska TE, Chaney RL, Chin M & Charvat I (2000) Effects of metal phytoextraction practices on the indigenous community of arbuscular mycorrhizal fungi at a metal-contaminated landfill. Appl. Environ. Microbiol. 66: 2526–2530

Pence NS, Larsen PB, Ebbs SD, Letham DL, Lasat MM, Garvin DF, Eide D & Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc. Natl. Acad. Sci. USA 25: 4956–4960

Peralta-Videa JR, Gardea-Torresdey JL, Gómez E, Tiemann KJ, Parsons JG & Carrillo G (2002) Effect of mixed cadmium, copper, nickel and zinc at different pHs upon alfalfa growth and heavy metal uptake. Environ. Pollut. 119: 291–301

Pichtel J, Kuroiwa K & Sawyerr HT (2000) Distribution of Pb, Cd and Ba in soils and plants of two contaminated soils. Environ. Pollut. 110: 171–178

Pickering IJ, Prince RC, George GN, Rauser WE, Wickramasinghe WA, Watson AA, Dameron CT, Dance IG, Fairlie DP & Salt DE (1999) X-ray absorption spectroscopy of cadmium phytochelatin and model systems. Biochim. Biophys. Acta-Prot. Struct. Mol. Enzymol. 1429: 351–364

Pickering IJ, Prince RC, George GN, Smith RD, George GN & Salt DE (2000) Reduction and coordination of arsenic in Indian mustard. Plant Physiol. 122: 1171–1177

Pilon-Smits E & Pilon M (2000) Breeding mercury-breathing plants for environmental cleanup. Trends Plant Sci. 5: 235–236

Pineros MA, Shaff JE & Kochian LV (1998) Development, characterization, and application of a cadmium-selective microelectrode for the measurement of cadmium fluxes in roots of *Thlaspi* species and wheat. Plant Physiol. 116: 1393–1401

Pulford ID & Watson C (2003) Phytoremediation of heavy metal-contaminated land by trees – a review. Environ. Int. 29: 529–540

Raskin I (1996) Plant genetic engineering may help with environmental cleanup (commentary). Proc. Natl. Acad. Sci. USA 93: 3164–3166

Raskin I, Smith RD & Salt DE (1997) Phytoremediation of metals: using plants to remove pollutants from the environment. Curr. Opin. Biotechnol. 8: 221–226

Rea PA (2003) Ion genomics. Nat. Biotechnol. 21: 1149–1151

Rengel Z (2000) Ecotypes of Holcus lanatus tolerant to zinc toxicity also tolerate zinc deficiency. Ann. Bot. 86: 1119–1126

Robinson B, Russell C, Hedley M & Clothier B (2001) Cadmium uptake by rhizobacteria: implications for New Zealand pastureland. Agri., Eco. & Environ. 87: 315–321

Rout GR, Samantaray S & Das P (1999) In vitro selection and biochemical characterisation of zinc and manganese adapted callus lines in *Brassica* spp. Plant Sci. 146: 89–100

Rubinelli P, Siripornadulsil S, Gao-Rubinelli F & Sayre RT (2002) Cadmium- and iron-stress-inducible gene expression in the green alga *Chlamydomonas reinhardtii*: evidence for H43 protein function in iron assimilation. Planta 215: 1–13

Rugh CL, Gragson GM, Meagher RB & Merkle SA (1998a) Toxic mercury reduction and remediation using transgenic plants with a modified bacterial gene. Hort. Science 33: 618–621

Rugh CL, Senecoff JF, Meagher RB & Merkle SA (1998b) Development of transgenic yellow poplar for mercury phytoremediation. Nat. Biotechnol. 16: 925–928

Rugh CL, Wilde HD, Stack NM, Thompson DM, Summers AO & Meagher RB (1996) Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. Proc. Natl. Acad. Sci. USA 93: 3182–3187

Sahi SV, Bryant NL, Sharma NC & Singh SR (2002) Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. Environ. Sci. Technol. 36: 4676–4680

Salt DE, Blaylock M, Kumar PBAN, Dushenkov V, Ensley BD, Chet I & Raskin I (1995a) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnol. 13: 468–475

Salt DE, Prince RC, Pickering IJ & Raskin I (1995b) Mechanisms of cadmium mobility and accumulation in Indian mustard. Plant Physiol. 109: 1427–1433

Salt DE, Prince RC, Baker AJM, Raskin I & Pickering IJ (1999) Zinc ligand in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Environ. Sci. Technol. 33: 713–717

Salt DE, Prince RC & Pickering IJ (2002) Chemical speciation of accumulated metals in plants: evidence from X-ray absorption spectroscopy. Microchemical J. 71: 255–259

Salt DE, Smith RD & Raskin I (1998) Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 643–668

Sanità di Toppi L & Gabbrielli R (1999) Response to cadmium in higher plants. Environ. Experim. Bot. 41: 105–130

Sauge-Merle S, Cuine S, Carrier P, Lecomte-Pradines C, Luu DT & Peltier G (2003) Enhanced toxic metal accumulation in engineered bacterial cells expressing *Arabidopsis thaliana* phytochelatin synthase. Appl. Environ. Microbiol. 69: 490–494

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J & Bleeker PM (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. J. Exp. Bot. 53: 1–12

Schneider T, Haag-Kerwer A, Maetz M, Niecke M, Povh B, Rausch T & Schübler A (1999) Micro-PIXE studies of elemental distribution in Cd-accumulating *Brassica juncea* L.

Nuclear Instr. Methods Phys. Res. Section B: Interaction with Materials and Atoms 158: 329–334

Schutzendubel A & Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53: 1351–1365

Schwartz C, Morel JL, Saumier S, Whiting SN & Baker AJM (1999) Root development of the zinc-hyperaccumulator plant *Thlaspi caerulescens* as affected by metal origin, content and localization in soil. Plant Soil 208: 103–115

Shanks JV & Morgan J (1999) Plant 'hairy root' culture. Curr. Opin. Biotechnol. 10: 151–155

Shann JR (1995) The role of plants and plant/microbial systems in the reduction of exposure. Environ. Health Perspect. 103: 13–15

Shen ZG, Zhao FJ & McGrath SP (1997) Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non-hyperaccumulator *Thlaspi ochroleucum*. Plant Cell Environ. 20: 898–906

Singh OV, Labana S, Pandey G, Budhiraja R & Jain RK (2003) Phytoremediation: an overview of metallic ion decontamination from soil. Appl. Microbiol. Biotechnol. 61: 405–412

Song W-Y, Sohn EJ, Martinoia E, Lee YJ, Yang Y-Y, Jasinski M, Forestier C, Hwang I & Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat. Biotechnol. 21: 914–919

Stomp AM, Han KH, Wilbert S, Gordon MP & Cunningham SD (1994) Genetic strategies for enhancing phytoremediation. Ann. NY Acad. Sci. 721: 481–491

Thomas JC, Davies EC, Malick FK, Endreszl C, Williams CR, Abbas M, Petrella S, Swisher K, Perron M, Edwards R, Ostenkowski P, Urbanczyk N, Wiesend WN & Murray KS (2003) Yeast metallothionein in transgenic tobacco promotes copper uptake from contaminated soils. Biotechnol. Prog. 19: 273–280

Tu C, Ma LQ & Bondada B (2002) Arsenic accumulation in the hyperaccumulator Chinese brake and its utilization potential for phytoremediation. J. Environ. Qual. 31: 1671–1675

United States Environmental Protection Agency (1992) Selection of control technologies for remediation of lead battery recycling sites. EPA/540/S-92/011. US Environmental Protection Agency, Office of Emergency and Remedial Response, Washington, DC, USA

United States Environmental Protection Agency (2000a) Electrokinetic and phytoremediation *in situ* treatment of metal-contaminated soil: state-of-the-practice. EPA/542. US Environmental Protection Agency, Office of Solid Waste and Emergency Response Technology Innovation Office, Washington, DC, USA

United States Environmental Protection Agency (2000b) Introduction to phytoremediation EPA/600/R-99/107. US Environmental Protection Agency, Office of Research and Development, Cincinnati, OH, USA

Vatamaniuk OK, Bucher EA, Ward JT & Rea PA (2002) Worms take the 'phyto' out of 'phytochelatins'. Trends Biotechnol. 20: 61–64

Visoottiviseth P, Francesconi K & Sridokchan W (2002) The potential of Thai indigenous plant species for the phytoremediation of arsenic contaminated land. Environ. Pollut. 118: 453–461

Wang QR, Cui YS, Liu XM, Dong YT & Christie P (2003) Soil contamination and plant uptake of heavy metals at polluted sites in China. J. Environ. Sci. Health Part A Tox. Hazard Subst. Environ. Eng. 38: 823–838

Wang QR, Liu XM, Cui YS, Dong YT & Christie P (2002a) Responses of legumes and non-legume crop species to heavy metals in soils with multiple metal contamination. J. Environ. Sci. Health Part A Tox. Hazard Subst. Environ. Eng. 37: 611–621

Wang Z, Shan X & Zhang S (2002b) Comparison between fractionation and bioavailability of trace elements in rhizosphere and bulk soils. Chemosphere 46: 1163–1171

Weber O, Scholz RW, Bühlmann R & Grasmück D (2001) Risk perception of heavy metal soil contamination and attitudes toward decontamination strategies. Risk Analysis 21: 967–977

Wenzel WW, Adriano DC, Salt D & Smith R (1999) Phytoremediation: A plant-microbe-based remediation system. In: SSSA (Ed), Bioremediation of Contaminated Soils (pp 457–508) Agronomy Monograph no. 37, SSSA, Madison, WI, USA

Whiting SN, Leake JR, McGrath SP & Baker AJM (2000) Positive response to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. New Phytol. 145: 199–210

Williams LE, Pittman JK & Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. Biochim. Biophys. Acta (BBA)-Biomembranes 1465: 104–126

Wong MH (2003) Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50: 775–780

Zhang W, Cai Y, Tu C & Ma LQ (2002) Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Sci. Total Environ. 300: 167–177

Zhao FJ, Lombi E, Breedon T & McGrath SP (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant Cell Environ. 23: 507–514

Zhao FJ, Hamon RE & McLaughlin MJ (2001) Root exudates of the hyperaccumulator *Thlaspi caerulescens* do not enhance metal mobilization. New Phytol. 151: 613–620

Zhu YL, Pilon-Smits EA, Jouanin L & Terry N (1999a) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiol. 119: 73–80

Zhu YL, Pilon-Smits EA, Tarun AS, Weber SU, Jouanin L & Terry N (1999b) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiol. 121: 1169–1178