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## REVIEWS AND ANALYSES

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### Phytoextraction of Toxic Metals: A Review of Biological Mechanisms

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#### ABSTRACT

Remediation of sites contaminated with toxic metals is particularly challenging. Unlike organic compounds, metals cannot be degraded, and the cleanup usually requires their removal. However, this energy-intensive approach can be prohibitively expensive. In addition, the metal removing process often employs stringent physicochemical agents which can dramatically inhibit soil fertility with subsequent negative impacts on the ecosystem. Phytoremediation has been proposed as a cost-effective, environmental-friendly alternative technology. A great deal of research indicates that plants have the genetic potential to remove many toxic metals from the soil. Despite this potential, phytoremediation is yet to become a commercially available technology. Progress in the field is hindered by a lack of understanding of complex interactions in the rhizosphere and plant-based mechanisms which allow metal translocation and accumulation in plants. In this paper, four research areas relevant to metal phytoextraction from contaminated soil are reviewed. The review concludes with an assessment of the current status of technology deployment and suggestions for future phytoremediation research.

PHYTOREMEDIATION, the use of plants for environmental restoration, is an emerging cleanup technology. To exploit plant potential to remediate soil and water contaminated with a variety of compounds, several technological subsets have been proposed. Phytoextraction is the use of higher plants to remove inorganic contaminants, primarily metals, from polluted soil. In this approach, plants capable of accumulating high levels of metals are grown in contaminated soil. At maturity, metal-enriched aboveground biomass is harvested and a fraction of soil metal contamination removed. Plants have a natural propensity to take up metals. Some, such as Cu, Co, Fe, Mo, Mn, Ni, and Zn, are essential mineral nutrients. Others, however, such as Cd and Pb, have no known physiological activity. Perhaps not surprisingly, phytoremediation as an environmental cleanup technology was initially proposed for the remediation of metal-contaminated soil (Utsunomyia, 1980; Chaney, 1983; Baker et al., 1991). The identification of metal hyperaccumulators, plants capable of accumulating extraordinarily high metal levels, demonstrates that plants have the genetic potential to clean up contaminated soil. As early as the 19th century, Baumann (1885), identified

plants capable of accumulating uncommonly high Zn levels. In 1935, Byers documented the accumulation of selenium in *Astragalus* spp. One decade later, Minguzzi and Vergnano (1948) identified plants capable of hyperaccumulating up to 1% Ni in shoots. Following the identification of these and other hyperaccumulator species, a great deal of research has been conducted to elucidate the physiology and biochemistry of metal hyperaccumulation in plants. Significant results have been obtained, and the understanding of metal accumulating mechanisms substantially advanced. However, a better understanding of the biological processes is needed if phytoextraction is to become a reliable, commercially available technology.

The success of phytoextraction, as an environmental cleanup technology, depends on several factors including the extent of soil contamination, metal availability for uptake into roots (bioavailability), and plant ability to intercept, absorb, and accumulate metals in shoots (Ernst, 1996). Ultimately, the potential for phytoextraction depends on the interaction between soil, metal, and plant. The complexity of this interaction, controlled by climatic conditions, argues against generic and in favor of a site specific phytoremediating approach. This underlines the importance of understanding the mechanisms and processes that govern metal uptake and accumulation in plants. In this review, four research areas, relevant to soil and plant interaction as it relates to metal phytoextraction, have been identified. The significance of these areas is briefly discussed below.

#### Soil Microorganisms and Metal Phytoextraction

Soil microorganisms have been shown to possess several mechanisms capable of altering metal bioavailability for uptake into roots. For example, microbes have been documented to catalyze redox reactions leading to changes in metal mobility in soil and propensity for uptake into roots. In addition, root mycorrhizal associations have been shown to affect the rate of metal uptake.

#### Metal Bioavailability for Uptake into Roots

For most metals, uptake into roots takes place from the aqueous phase. Strong binding to soil particles and/or precipitation renders a significant soil metal fraction insoluble, and largely unavailable for plant uptake. Low soil bioavailability is a major factor limiting the potential for phytoextraction of significant metal contaminants such as lead. A major objective of current phytoremedi-

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ation research is to induce lead desorption from soil matrix into solution, and increase propensity for uptake into roots.

### Plant-Based Remedial Mechanisms

The identification of metal hyperaccumulator species demonstrates that plants possess the genetic potential to remove toxic metals from contaminated soil. Understanding the plant-based remedial mechanisms is important for several reasons. For example, the elucidation of these mechanisms may provide clues for optimizing the effectiveness of phytoremediation with appropriate agronomic practices. In addition, the identification and biochemical characterization of the remedial mechanisms are necessary preliminary steps to isolating plant genes responsible for the expression of the remediating phenotype. The identification and isolation of these genes may open the opportunity to use biotechnology to ameliorate plants for environmental cleanup.

#### Improving Plants for Environmental Remediation

In general, plants express an incomplete set of remediating features. For example, most of the metal hyperaccumulators are small and slow growing. Conventional breeding and biotechnology have been used to correct these shortcomings by transferring desired traits from metal hyperaccumulator plants to selected high-biomass-producing nonaccumulator species.

In the following section, current research status in the areas identified above is reviewed and summarized.

### CURRENT RESEARCH STATUS

#### Soil Microorganisms and Metal Phytoextraction

Several lines of evidence suggest that soil microorganisms possess mechanisms capable of altering environmental mobility of metal contaminants with subsequent effects on the potential for root uptake. For example, a strain of *Xanthomonas maltophyla* was shown to catalyze the reduction and precipitation of highly mobile  $\text{Cr}^{6+}$  to  $\text{Cr}^{3+}$ , a significant less-mobile and environmentally less-hazardous compound (Blake et al., 1993). The same strain was also found to induce the transformation of other toxic metal ions including  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Au}^{3+}$ ,  $\text{Te}^{4+}$ ,  $\text{Ag}^+$ , and oxyanions, such as  $\text{SeO}_4^-$ . Strains of *Escherichia coli* and *Pseudomonas putida* with similar characteristics were subsequently identified (Shen and Wang, 1993, 1994, 1995; Wang and Shen, 1995, 1997; Wang and Changsong, 1995; Chirwa and Wang, 1997; Wang and Chirwa, 1998; Park et al., 1999). These properties prompted some scientists to propose the use of selected microorganisms to remove toxic metals from wastewater (Blake et al., 1993). Emphasizing a different mechanism, a recent study revealed that As mobility can be enhanced by the activity of dissimilatory nonreducing bacterium *Shewanella alga* (Cummings et al., 1999). Strain BrY of this bacterium was shown to induce As mobilization from sorption sites within whole sediments. Ionic mercury ( $\text{Hg}^{2+}$ ) which is susceptible to methyl-

tion and subsequent generation of methyl-mercury, a highly neurotoxic compound, was shown to be reduced by some bacteria to  $\text{Hg}^0$ , a volatile element which poses less environmental risk (EPA-452/R-97-003). Recently, Crusberg (1998) proposed the investigation of the fungus *Penicillium ochro-chloron* for the ability to precipitate a variety of heavy metals including Cu, Ni, Pb, and Cd. In addition, soil microorganisms have been shown to exude organic compounds which stimulate bioavailability and facilitate root absorption of a variety of metal ions including  $\text{Fe}^{2+}$  (Crowley et al., 1991; Bural et al., 2000),  $\text{Mn}^{2+}$  (Barber and Lee, 1974), and possibly  $\text{Cd}^{2+}$  (Salt et al., 1995a).

Microbes are also known to alter chemical properties of the rhizospheric soil with subsequent effects on the environmental mobility of metal contaminants. For example chemolithotrophic bacteria have been shown to enhance environmental mobility of metal contaminants via soil acidification, or, in contrast, to decrease their solubility due to precipitation as sulfides (Kelley and Tuovinen, 1988).

It is generally accepted that under natural conditions a majority of plants have mycorrhizae (Smith and Reed, 1997). Fungal symbiotic associations have the potential to enhance root absorption area, and stimulate the acquisition of plant nutrients including metal ions (Khan et al., 2000). For example, the length of mycorrhizae associated with pine and willow seedlings was estimated to be two orders of magnitude greater than the length of host root system (Jones et al., 1990; Rousseau et al., 1994). In addition, fungal associations were shown to enhance root absorption area up to 47-fold (Smith and Read, 1997). Mycorrhizae has been reported in plants growing on heavy metal-contaminated soil (Shetty et al., 1994; Chaudry et al., 1998, 1999). In addition, arbuscular mycorrhizal fungi are known to colonize ferns (Sharma, 1998), suggesting a possible role of mycorrhizal associations in the recently reported arsenic hyperaccumulation (Ma et al., 2001). Among mycorrhizal fungal species identified in metal rich soils are *Glomus* spp., *Gigaspora* spp., and *Eutrophosphora* spp. (Raman and Sambandan, 1998; Chaudry et al., 1999; Pawlowska et al., 1996). There are contradictory reports as to the effect of mycorrhizae on metal uptake. Weissenhorn and Leyval (1995) and Joner and Leyval (1997) indicated that Cd is sequestered in the hyphae of *Glomus mosseae* with subsequent restriction of metal transfer from the fungus to the root of subterranean clover plants. An inhibition of Zn and Cu accumulation in mycorrhizal plants was also reported by Schuepp et al. (1987) and Heggo et al. (1990). Binding to the cell wall of the hyphae has been proposed as a mechanism by which ectomycorrhizal fungi protect the plant from metal toxicity (Galli et al., 1994). In addition, Turnau (1998) reported that Zn was deposited as crystalloids within the fungal mycelium and cortical cells of *Euphorbia cyparissias* mycorrhizal roots. However, other studies could not confirm the inhibition of metal uptake and accumulation in mycorrhizal plants. On the contrary, Zn concentration in pine tree was stimulated by *Thelephora terrestris* (Colpaert and Vannasche, 1992). Furthermore, tolerance of *Picea abies*

to Cd conferred by *Paxillus involutus* could not be related to an inhibition of Cd uptake (Goldbold et al., 1998). In addition to the effect on metal root uptake, mycorrhizae has also been reported to affect metal transport within plant. For example, mycorrhizae has been shown to alter the pattern of Zn translocation from root to shoot in the grass *Andropogon gerardii* (Shetty et al., 1994). An inhibition of Zn translocation to shoots was also reported in mycorrhizal maize seedlings (Khan et al., 2000). However, this effect appeared to be species specific since mycorrhizae did not affect Zn translocation pattern in *Festuca arrundinaceae* (Shetty et al., 1994). In support of this species-specific effect, Dhillon (1992) has indicated that "host preference" is an important characteristic of the symbiotic association.

In a greenhouse study, Zn accumulation in roots was lower in plants inoculated with mycorrhizal fungi although the effect of mycorrhizae on the rate of Zn uptake into root was not clear (Banks et al., 1994). These results suggest that one way of increasing the rate of metal uptake is to minimize root colonization by mycorrhizae. Recently, Burke et al. (2000) showed that soil application of the fungicide Benomyl significantly decreased maize root colonization by arbuscular mycorrhizae. This treatment was associated with an increase in Pb concentration in shoots. However, total Pb removal was inhibited due to a reduction in shoot biomass caused by fungicide application.

A great deal of data indicates that soil microorganisms have the potential to alter the rate of metal uptake and accumulation in plants. Thus, microbes have been shown to catalyze redox transformations leading to changes in soil metal bioavailability and potential for phytoextraction. In addition, the exudation of organic compounds from roots has been shown to affect root absorption of certain metals. The effect of mycorrhizal associations on metal root uptake is not clear and appears to be metal and plant specific. The bulk of evidence tends to indicate an inhibition of metal uptake by mycorrhizae. However, it is possible that adapted mycorrhizal fungi may play an important role in hyperaccumulation of some metals and metalloids.

### **Metal Bioavailability for Uptake into Roots**

In soil, metals exist as a variety of chemical species in a dynamic equilibrium governed by soil physical, chemical, and biological properties (Chaney, 1988). The kinetics of this interaction, which ultimately dictates the extent of metal bioavailability, is poorly understood. In general, only a fraction of soil metal is readily available (bioavailable) for plant uptake. The bulk of soil metal is commonly found as insoluble compounds unavailable for transport into roots. The investigation of metal bioavailability routinely requires soil extraction studies. In general, aqueous extraction provides an estimate of the amount of bioavailable metal in the soil solution. Estimates of total bioavailable metal (which also includes metal ions bound to soil exchange sites) are obtained by extracting the soil with organic compounds such as  $\text{NH}_4$ -acetate (Ernst, 1974) or DTPA-TEA (diethylene-

triamine-pentaacetic acid-triethanolamine) (Lindsay and Norvell, 1978).

With the exception of mercury, metal uptake into roots occurs from the aqueous phase. In soil, some metals, such as Zn and Cd, occur primarily as soluble or exchangeable, readily bioavailable form. Others, such as, Pb occur as insoluble precipitates (phosphates, carbonates, and hydroxy-oxides) which are largely unavailable for plant uptake (Pitchel et al., 1999). Binding and immobilization within the soil matrix can significantly restrict the potential for metal phytoextraction. Despite the adverse effect on metal root uptake, soil inactivation with chemical amendments has been proposed as a temporary solution for the remediation of Pb-contaminated soil (Cunningham and Berti, 1993; Cotter-Howells and Caporn, 1996). It should be added that although in-place inactivation has the potential to temporarily reduce the risk of spreading metal contamination (Berti and Cunningham, 1993, 1997), soil-sequestered Pb may act as a long-term pollution source posing a risk to humans and animals due to accidental ingestion of contaminated soil or inhalation of airborne contaminated dust. Pierzynski and Schwab (1993) investigated the effect of chemical amendments on the potential for phytoextraction of several toxic metals including Cd, Pb, and Zn. They showed that addition of limestone, cattle manure, and poultry litter to soil significantly reduced Zn bioavailable fraction. This result correlated well with a reduction of soybean tissue Zn concentration in limestone treatment compared with control. However, Cd and Pb tissue concentrations was not significantly affected by limestone or manure application, suggesting that the effect of soil amendment on bioavailability is metal specific.

Plants possess highly specialized mechanisms to stimulate metal bioavailability in the rhizosphere, and to enhance uptake into roots (Romheld and Marschner, 1986). Thus, grass species have been documented to exude a class of organic compounds termed siderophores (mugineic and avenic acids) capable of enhancing the availability of soil iron for uptake into roots (Fushiya et al., 1982; Takagi et al., 1984). Dicotyledonous species facilitate iron uptake by acidifying the rhizosphere via  $\text{H}^+$  extrusion from roots. Acidic environment stimulates the reduction of ferric to ferrous iron which is readily taken up by plants (Chaney et al., 1972; Bienfait et al., 1982).

Fan et al. (1997, 1999), investigated the effect of humic substances on root exudation of organic compounds and metal uptake. Exudation of major ligands including deoxymugineic acid, malate, and acetate from wheat roots was inhibited by the addition of humic substances. These authors also noticed that reduction in acetate exudation was correlated to lower Fe and Zn concentration in roots and Cu, Fe, Mn, Ni, and Zn in shoots.

Lead is a major metal contaminant notorious for posing a significant risk to humans, especially children. For example, it has been estimated that in the USA alone lead poisoning affects more than 800 000 children between the age of one and five (Pirkle et al., 1998). The potential for Pb phytoextraction is limited primarily due to low soil mobility and little propensity for lead uptake

into roots. Recent research has focused on artificially inducing Pb desorption from soil solids. Several chelating agents have been tested for this purpose. Addition of EDTA (ethylene-diamine-tetraacetic acid), at a rate of 10 mmol/kg soil, stimulated Pb accumulation in maize to levels as high as 1.6% of shoot dry weight (Blaylock et al., 1997). Similar results have been reported by Vassil et al. (1998). A surge in pea Pb accumulation was also associated with the addition of synthetic chelates (Huang and Cunningham, 1996). Recently, Kirkham (2000) employed a sequential extraction to investigate the effect of EDTA on the solubility of Pb associated with different soil fractions. The results indicated that soil EDTA addition mobilized Pb associated with the ion-exchangeable and carbonate soil fractions. Lead retained in the oxide and the organic fraction was less affected by EDTA addition (Elless and Blaylock, 2000). Huang et al. (1997) indicated that a variety of synthetic chelates have a potential to induce Pb desorption from the soil matrix. Their effectiveness, in decreasing order, was EDTA > HEDTA > DTPA > EGTA > EDDHA. Unfortunately, highly soluble Pb-EDTA easily percolates through soil profile, posing a high risk of groundwater contamination (Wu et al., 1999). The same authors indicated that the application of HBED (N,N-di(2-hydroxybenzyl)ethyleneamide N,N-prime-diacetic acid) resulted in a greater stimulation of Pb accumulation in maize roots than EDTA. The effect of HBED application on Pb translocation from root to shoot was less clear. In a recent field study, application of nitriloacetate (NTA) and elemental sulfur increased the solubility of Zn, Cd, and Cu by as much as 21-, 58-, and 9-fold, respectively (Kayser et al., 2000). However, plant accumulation of these metals increased by a factor of 2 to 3 only. An interesting effect was reported by Robinson et al. (1997) who indicated that EDTA addition increased soil soluble Ni but decreased metal uptake by *Berkheya codii*. Lasat et al. (1998a) investigated the effect of ammonium ion addition on radiocesium bioavailability and uptake in three plant species. Addition of  $\text{NH}_4^+$  released more  $^{137}\text{Cs}$  from soil solids into solution. Mobilized  $^{137}\text{Cs}$ , however, quickly percolated through soil before being absorbed by roots. These results clearly illustrate the potential risk of enhancing the solubility of soil contaminants with chemical additives and the need to better manage and contain the spread of pollution. Research has been conducted to maximize metal bioavailability while minimizing the environmental risk posed by chelate application. Elless and Blaylock (2000) suggested that for Pb removal, an amendment formulation combining lower EDTA doses and surfactants may be an attractive alternative to higher rates of soil EDTA application. In support of this, Mulligan et al. (2001) have recently pointed out that biosurfactants have the potential to enhanced metal bioavailability in contaminated soil and sediments. Wu et al. (1999) showed that the risk of pollution spread can be significantly reduced by applying EDTA to plants transplanted into contaminated soil. These results suggest that with innovative agronomic practices notable improvements over current chelate-assisted phytoextraction may be possible. Blay-

lock et al. (1997) indicated that in addition to Pb, bioavailability of other metals including Cd, Cu, Ni, and Zn can be enhanced by the application of synthetic chelates. For example, EGTA (ethylenebis [oxyethylenetrinitrilo] tetraacetic acid) has been shown to have a high affinity for  $\text{Cd}^{2+}$ , but not for  $\text{Zn}^{2+}$ . EDTA, HEDTA, and DTPA are selective for  $\text{Zn}^{2+}$ . The addition of EDTA, citric and oxalic acid to a contaminated soil dramatically increased (>200-fold)  $\text{Cr}^{3+}$  uptake and accumulation in roots and shoots (Shahandeh and Hosner, 2000). Chen and Hong (1995) indicated that acetamido-iminodiacetic acid stimulated Pb bioavailability while S-carboxymethylcysteine was effective for Cu (Hong et al., 1995). Phytoextraction of precious metals was investigated by Anderson et al. (1999). They indicated that up to 57 mg/kg Au was accumulated by Indian mustard grown in soil treated with ammonium thiocyanate. The same authors have reported unusual high accumulations of thallium; 0.4%, and 1.5%, on a dry weight basis, in *Iberis intermedia* and *Biscutella laevigata*, respectively.

The potential for phytoextraction of several major metal contaminants including Pb is adversely affected by metal adsorption to soil solids and/or precipitation as insoluble compounds. Addition of synthetic chelates has been shown to stimulate the release of metals into soil solution and enhance the potential for uptake into roots. In the case of Pb, however, EDTA addition to the soil has been documented to increase the risk of spreading contamination due to high solubility of Pb-chelate complex. Recent research aims at eliminating this risk by implementing alternative chelate formulations and innovative agronomic practices.

### Plant-Based Remedial Mechanisms

Interest in Phytoextraction has grown significantly following the identification of metal hyperaccumulator plants. Hyperaccumulators are species capable of accumulating metals at levels 100-fold greater than those typically measured in shoots of the common nonaccumulator plants. Thus, a hyperaccumulator will concentrate more than 10 ppm Hg; 100 ppm Cd; 1000 ppm Co, Cr, Cu, and Pb; 10 000 ppm Zn, and Ni. To date, approximately 400 plant species from at least 45 plant families have been reported to hyperaccumulate metals (Baker et al., 2000). Most of these species accumulate Ni, about 30 accumulate either Co, Cu, and Zn, even fewer accumulate Mn and Cd, and there are no known Pb hyperaccumulators. Recently, a fern (*Pteris vitatta*), collected from an abandoned wood preservation site has been shown to accumulate as much as 14 500 ppm arsenic in fronds without showing symptoms of toxicity (Ma et al., 2001). These results suggest that phytoremediation of As-contaminated sites is feasible. Several hyperaccumulator species and their metal accumulation potential are listed in Table 1.

Possibly, the best-known metal hyperaccumulator is *Thlaspi caerulescens* (Alpine pennycress). While most plants exhibit toxicity symptoms at Zn concentrations of about 100 ppm, *T. caerulescens* was shown to accumu-

**Table 1. Several metal hyperaccumulator species and their bioaccumulation potential.**

Plant species	Metal	Leaf content	Reference
		ppm	
<i>Thlaspi caerulescens</i>	Zn:Cd	39 600:1 800	Reeves and Brooks (1983); Baker and Walker (1990)
<i>Ipomea alpina</i>	Cu	12 300	Baker and Walker (1990)
<i>Sebertia acuminata</i>	Ni	25% by wt. dried sap	Jaffre et al. (1976)
<i>Haumaniastrum robertii</i>	Co	10 200	Brooks (1977)
<i>Astragalus racemosus</i>	Se	14 900	Beath et al. (1937)

late up to 26 000 ppm without showing any injury (Brown et al., 1995a). In addition, this species extracted up to 22% of soil exchangeable Cd from a contaminated site (Gerrard et al., 2000) and showed remarkable Cd tolerance (Schat et al., 2000; Escarre et al., 2000; Lombi et al., 2000). Because of these properties, *T. caerulescens* represents an excellent experimental system for studying the mechanisms of metal uptake, accumulation, and tolerance as they relate to metal phytoextraction (Lasat et al., 1996, 1998b, 2000; McGrath et al., 1997; Shen et al., 1997). In addition, other *Thlaspi* spp. and *Brassica juncea* have been used as a model system to investigate the physiology and biochemistry of metal accumulation in plants (Salt et al., 1995b, 1997, 1999; Krämer et al., 1996, 1997).

Transport across root cellular membrane is an important process which initiates metal absorption into plant tissues. The electrical charge prevents metal ions from diffusing freely across the lipophilic cellular membranes into the cytosol. Therefore, ion transport into cells must be mediated by membrane proteins with transport functions. Root uptake kinetics have been investigated for a variety of metal ions including Cd<sup>2+</sup> (Cohen et al., 1998; Hart et al., 1998), Cu<sup>2+</sup> (Thornton, 1991), and Zn<sup>2+</sup> (Santa Maria and Cogliatti, 1988; Vázquez et al., 1994). A comprehensive characterization of Zn<sup>2+</sup> transport and accumulation in related hyperaccumulator and nonaccumulator plant species was reported by Lasat et al. (1996, 1998b). These authors indicated that in *T. caerulescens*, Zn hyperaccumulation was correlated with a stimulation of Zn influx into root cells. In addition, an alteration of Zn intracellular transport was also documented in *T. caerulescens*. Lasat et al. (1998b) showed that sequestration in the root vacuole prevented Zn translocation from root to shoot in *T. arvense* (a related nonaccumulator), whereas in *T. caerulescens* the mechanism of vacuolar sequestration is disabled allowing Zn translocation and hyperaccumulation in leaves.

Accumulation of elevated levels of metal ions is phytotoxic. Plants have developed strategies to defend against this potential stress. Metal tolerance may result from two basic strategies: metal exclusion and metal detoxification (Baker, 1981). The excluders prevent metal uptake into roots avoiding translocation and accumulation in shoots (De Voss et al., 1991). Excluders have a low potential for metal extraction, but they can be used to stabilize the soil, and avoid further contamination spread due to erosion. Such a species is *Agrostis tenuis* (a pseudometallophyte) which avoids Cd, Cu, Pb, and Zn uptake by precipitating the metal in the rhizosphere (Dahmani-Muller et al., 2000). In contrast, hyperaccumulators absorb high levels of metals in cells.

Such elevated metal concentrations are toxic to common nonaccumulator plants. A primary effect of metal toxicity is the inhibition of a number of cytoplasmic enzymes (Assche and Clijsters, 1990). Therefore the homeostasis of metal ions in the cytoplasm is essential for avoiding toxicity (Meharg, 1994). Metal tolerance is a major characteristic of hyperaccumulator species. In fact, in some species, such as the Ni hyperaccumulator *T. goesingense*, hyperaccumulating phenotype is exclusively conferred by exceptionally high metal tolerance (Krämer et al., 1997). Early studies suggested that following uptake intracellular nickel is detoxified via binding to low molecular weight organic compounds (<10 kD) (Lee et al., 1977). More recently, Krämer et al. (2000) showed that in the Ni-hyperaccumulator *T. goesingense*, intracellular Ni is predominantly localized in the vacuole as a Ni-organic acid complex, possibly citrate whereas cytoplasmic Ni is detoxified via binding to histidine (Persans et al., 1999). Zinc is another metal for which several hyperaccumulator species have been identified. Brune et al. (1994) discussed the mechanism of Zn tolerance in plants and proposed five putative mechanisms for regulation of cytoplasmic Zn: 1) low uptake across the plasma membrane, 2) sequestration in a subcellular organelle, 3) precipitation as insoluble salts, 4) complexation to low molecular weight organic ligands, and, 5) active extrusion across the plasma membrane into the apoplast. A considerable body of evidence argues against hypotheses: 1, 3, and 5 as the mechanism of Zn tolerance in *T. caerulescens* (Lasat et al., 1996; Tolrà et al., 1996; Shen et al., 1997; Zhao et al., 1998). In contrast, there is substantial evidence supporting the mechanism of cellular sequestration. Several transporters have been shown to mediate Zn fluxes across cellular membranes (Paulsen and Saier, 1997) including tonoplast (MacDiarmid et al., 2000; Lyons et al., 2000). In yeast, overexpression of membrane proteins MTP's (Metal Tolerance Proteins) from *T. goesingense* was shown to confer resistance to Cd, Co, Ni, and Zn, possibly due to transport into the vacuole (Persans et al., 2000). A family of transporters, similar to *Nramp* transporters identified in animals, bacteria and yeast, with a possible role in Cd transport was identified in *Arabidopsis* although the cellular localization of these transporters is not known (Thomine et al., 2000). Furthermore, metal compartmentalization in the vacuole has been previously documented (Vázquez et al., 1994; Küpper et al., 1999). Vázquez et al. (1994) proposed that following transport across the tonoplast, Zn is precipitated in the vacuole of epidermal and subepidermal cells of the *T. caerulescens*' leaf. Recent results from single-cell sap extraction studies confirmed this observation (Küpper et al., 1999).

Other studies, however, suggested that most of Zn accumulated in *T. caerulea* leaves is water soluble (Tolrà et al., 1996; Zhao et al., 1998). Ernst (1975), and Mathys (1975) suggested that a Zn-malate complex, formed in the cytoplasm, shuttles Zn into the vacuole where it dissociates. In the vacuole, zinc was proposed to be complexed and stored as oxalate (which is a much stronger Zn ligand at the acidic pH of the vacuole). Subsequent studies, however, have not confirmed this hypothesis. For example, Tolrà et al. (1996) found constitutively high levels of malate in shoots of *T. caerulea*, although a clear correlation to zinc accumulation was not observed. Shen et al. (1997) also found very high concentrations of malate in *T. caerulea* shoots. However, high malate concentrations were also measured in *T. ochroleuca*, which the authors used as a reference nonaccumulator species. This result argues against a role of malate in Zn tolerance as proposed by Mathys (1977). More recently, precipitation in the vacuole as Zn-phytate has been proposed to account for Zn inactivation in the vacuole (Van Steveninck et al., 1990).

It has been documented that in animals and certain fungi metal uptake induces the production of small cysteine-rich proteins known as metallothioneins (MTs) (Hamer, 1986). In plants, MT proteins have been identified only in wheat (Lane et al., 1987) and *Arabidopsis* (Murphy et al., 1997). Although detection of plant MTs has been problematic, evidence suggests that they have the ability to bind heavy metals. For example, Murphy and Taiz (1995) have shown that in *Arabidopsis* MT gene expression can be modulated in response to copper. In addition, expression of *Arabidopsis* MTs, restored copper tolerance in a copper-sensitive *S. cerevisiae* mutant lacking the MT encoding *cup1* gene (Zhou and Goldsberg, 1994). In a similar approach, modified yeast cells were employed to demonstrate that MTs are also involved in Zn tolerance (Robinson et al., 1996). Despite these significant results, *Arabidopsis* MTs could not be isolated in protein extracts obtained from the transformed yeast strains emphasizing the difficulty of detecting plant MTs. It should be added that the detection of MT proteins in plant tissues has relied mostly on the analysis of MT RNA levels. However, this approach fails to address the possible involvement of postranscriptional processes in the function of MT genes (Cobbett and Goldsbrough, 2000).

Accumulation of heavy metals in plants has been shown to induce the production of phytochelatins (PCs), a family of thiol-rich peptides (Steffens, 1990; Rauser, 1995). The synthesis of phytochelatins has been documented to be induced by a variety of metals. However, PCs have been shown to be primarily involved in Cd and Cu tolerance (Rauser, 1990; Ow, 1996). In support of this, Chen and Goldsbrough (1994) found an increased activity of gamma-glutamyl-cysteine synthetase activity in tomato cells selected for cadmium tolerance. Howden and Cobbett (1992) and Howden et al. (1995a, 1995b), have isolated *A. thaliana* mutants with increased sensitivity to Cd. These *cad1* mutants, deficient in PC synthesis, showed greatly reduced levels of PC synthase

activity, a major enzyme of the PC biosynthetic pathway. By screening plant genes for metal tolerance, a wheat cDNA, *TaPCSI*, was identified whose expression in *Saccharomyces cerevisiae* significantly stimulated cadmium tolerance (Clemens et al., 1999; Vatamaniuk et al., 1999). In addition, cells expressing *PCS* genes accumulated more Cd than untransformed cells. Possibly, PCs act as a cytoplasmic scavenger and carrier for Cd transport into the vacuole where it is stored as a stable compound. Ortiz et al. (1995) demonstrated that *hmt1* is a gene encoding a transporter which mediates the transport of the Cd-PC complex into the vacuole of *Schizosaccharomyces pombe*. In support of this, yeast mutants lacking the ability to express *hmt1* were Cd sensitive. A tonoplast transporter capable of transporting both PCs and Cd-PC complexes from the cytoplasm into the vacuole has also been identified in plants (Salt and Rauser, 1995). Bae and Mehra (1997), studied the metal-chelating characteristics of a synthetically constructed PC analogue peptide (Glu-Cys)<sub>2</sub> and found that (Glu-Cys)<sub>2</sub> exhibited a Cd-binding stoichiometry of 0.5 Cd atom per peptide molecule. The stoichiometry for Hg<sup>2+</sup>, and Pb<sup>2+</sup> was one metal atom for each (Glu-Cys)<sub>2</sub> molecule. Salt et al. (1995b) investigated Cd accumulation in Indian mustard and found that high Cd levels were associated with a rapid accumulation of phytochelatins in roots. A recent study suggested that phytochelatins may also be involved in arsenic detoxification (Schmoger et al., 2000).

To avoid toxicity, plants have also been documented to catalyze redox reactions and alter the chemistry of metal ions. For example, Lytle et al. (1998) showed that from a solution supplemented with toxic Cr<sup>6+</sup>, water hyacinth (*Eichornia crassipes*) accumulated nontoxic Cr<sup>3+</sup> in roots and shoots. The Cr<sup>6+</sup> to Cr<sup>3+</sup> reduction apparently took place in the fine lateral roots. The reduced ion (Cr<sup>3+</sup>) was subsequently translocated to leaf tissues. DeSouza et al. (2000) investigated Se accumulation and showed that in non-accumulator plants, following uptake via a sulfate transporter, selenate is reduced to selenite, process catalyzed by the enzyme ATP sulfurylase. However, the evidence does not support the existence of a similar process in Se hyperaccumulator species. Pickering et al. (2000) indicated that in *B. juncea*, As<sup>5+</sup> is transported in roots as a phosphate analogue which is subsequently reduced to As<sup>3+</sup> in shoots where it is primarily stored as As<sup>3+</sup>-tris-thiolate.

There is evidence suggesting that biochemical mechanism that confers hyperaccumulating properties is both species and metal specific. For example, in *T. goevingense* high Ni tolerance was shown to primarily account for the expression of the Ni hyperaccumulator phenotype (Krämer et al., 1997). However, in *T. caerulea*, stimulated transport into the cells was also shown to play a role in Zn hyperaccumulation. In support of this, Lasat et al. (2000) and Pence et al. (2000), demonstrated that the expression of a gene, *ZNT1*, encoding a Zn transporter was stimulated in *T. caerulea* compared with a nonaccumulator relative, *T. arvense*. *ZNT1* was shown to be a member of the *ZIP* family of plant micronutrient transporters (Eide et al., 1996; Grotz et al., 1998; Gueriot, 2000). Lasat et al.

(2000) and Pence et al. (2000) further speculated that high expression of *ZNT1* in *T. caeruleus* is related to a mechanism that allows detoxification of ionic zinc to a physiologically less active compounds which is subsequently stored in the leaf cells of the hyperaccumulator species.

The genetic control of metal hyperaccumulation in plants is not well understood. For example, analyses of the heritability of Zn hyperaccumulation in *T. caeruleus* yielded inconclusive results (Pollard and Baker, 1996; Pollard et al., 2000). Early work suggested that the genetic control of metal tolerance is complex (Antonovics et al., 1971). However, more recent studies have indicated that metal tolerance is regulated by few major genes (Macnair, 1993). Smith and Macnair (1998) conducted a genetic analysis of copper tolerance with Cu-tolerant and -susceptible lines of *Mimulus guttatus*, and showed that the difference in tolerance was caused by a modifier gene which is active only when the gene for tolerance is present. However, the two genes acted in conjunction to define one independent mechanism. Recently, Macnair et al. (2000) crossed the tolerant Zn hyperaccumulator *A. halleri* and the nontolerant nonaccumulator relative *A. petrea*, and concluded that the two characters are genetically independent. Their data also suggested that Zn tolerance is controlled by a single major gene. The observation that tolerance to toxic metals is regulated by one or few major genes suggests that biotransformation of selected crop species for tolerance, and ultimately superior metal extraction potential is feasible (Macnair et al., 2000).

Understanding the physiology and biochemistry of metal accumulation in plants is important for several reasons. Firstly, this would allow the identification of agronomic practices capable of optimizing the potential for phytoextraction. In addition, understanding the physiology and biochemistry of metal tolerance is needed in order to identify and isolate genes responsible for the expression of the hyperaccumulating phenotype. A significant body of evidence suggests that metal tolerance is an important feature of hyperaccumulator plants. Research data indicates that metal tolerance is regulated by few major genes. This provides hope that biotechnology may be used to engineer more efficient hyperaccumulator plants.

### Improving Plants for Environmental Remediation

To improve the potential for metal phytoextraction, Brown et al. (1995b) proposed the transfer of the hyperaccumulator phenotype from small and slow growing hyperaccumulator species (Ebbs et al., 1997) to fast growing, high biomass-producing nonaccumulator plants. To achieve this goal, Cunningham and Ow (1996), and Chaney et al. (2000) suggested the use of conventional breeding to improve plants for metal extraction. In this approach, however, the potential for success can be limited because of anatomical constraints that can severely restrict sexual compatibility between species (Ow, 1996). As an alternative, biotechnology offers the

opportunity for direct gene transfer, thus circumventing limitations imposed by sexual incompatibility.

Biotechnology has been employed to identify Pb-tolerant mutants from mutagenized *Arabidopsis* populations (Chen et al., 1997) providing an opportunity to isolate genes for Pb tolerance. Screening of mutagenized populations of *Arabidopsis* has also resulted in the identification of mutants capable of accumulating elevated levels of Mn (Delhaize et al., 1993) and P (Delhaize and Randall, 1995). Possibly, the most spectacular application of biotechnology for environmental restoration has been the bioengineering of plants capable of removing methyl-mercury from the contaminated soil (Rugh et al., 1996; Heaton et al., 1988; Pilon-Smits and Pilon, 2000). Methylmercury, a strong neurotoxic agent, is biosynthesized in Hg-contaminated soils. To detoxify this compound, transgenic plants have been engineered to express modified bacterial genes *merB* and *merA*. In transformed plants, *merB* catalyzes the protonolysis of the carbon-mercury bond with the release of  $Hg^{2+}$ , a less mobile mercury species. Subsequently, *merA* converts  $Hg^{2+}$ , taken up by roots to  $Hg^0$ , a less toxic volatile element which is released into the atmosphere. Rugh et al., (1998), genetically modified yellow poplar trees. Genetically modified plantlets germinated and grew vigorously in media normally containing toxic levels of  $Hg^{2+}$  and released elemental mercury at approximately 10 times the rate of untransformed plants. In a recent study, Bizily et al. (2000), engineered transgenic *A. thaliana* plants to express bacterial genes, *merA* and *merB*. Modified plants were able to grow on media containing 50-fold higher methylmercury concentrations than wild type plants and up to 10-fold higher concentrations than plants that expressed *merB* alone. To improve the potential for phytoextraction of Se-contaminated soil, Pilon-Smits et al. (1999) overexpressed an ATP-sulfurylase (APS) gene, cloned from *A. thaliana* (Leustek et al., 1994), in Indian mustard. The transgenic plants had fourfold higher APS enzymatic activity, and accumulated 3 times more Se than the wild type. In a recent study, tomato plants have been modified to express the bacterial gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. These transgenic plants showed enhanced metal tolerance and were able to acquire greater metal levels (Cd, Co, Cu, Mg, Ni, Pb, and Zn) than untransformed plants (Grichko et al., 2000). Furthermore, when *PsMTA* gene encoding the metallothionein-like compounds in peas was expressed in *A. thaliana*, more Cu was accumulated in the roots of the transformed than control plants (Evans et al., 1992). Enhanced accumulation was attributed primarily to a stimulation of Cu tolerance in transgenic plants since copper uptake and translocation was unaffected by the genetic manipulation. To investigate the importance of glutathione and PC for Cd tolerance, Indian mustard was genetically engineered to overexpress the *E. coli gshI* gene encoding  $\gamma$ -glutamylcysteine synthetase (Zhu et al., 1999). Concentration of Cd in the shoots of transgenic plants was up to 90% greater than wild-type plants. In a different study, plants engineered to overexpress the *A. thaliana* gene (*ZAT*) related to putative zinc-trans-

**Table 2. Current research status, readiness for commercialization, and regulatory acceptance of phytoremediation for several metal and metalloid contaminants.**

	Contaminant							
	Ni	Co	Se	Pb	Hg	Cd	Zn	As
Commercial readiness <sup>†</sup>	4	4	4	4	3	2	3	1
Regulatory acceptance <sup>‡</sup>	Y	Y	N	Y	N	Y	Y	N

<sup>†</sup> Rating: 1, basic research underway; 2, laboratory stage; 3, field deployment; 4, under commercialization.

<sup>‡</sup> Regulatory acceptance: Y, yes; N, no.

porter genes (*ZnT*) from animals exhibited enhanced Zn resistance and accumulation in roots (van der Zaal et al., 1999).

To improve the potential for metal phytoextraction, Brewer et al. (1999) generated somatic hybrids between the Zn hyperaccumulator *T. caerulescens* and *B. napus*. The hybrids accumulated high Zn levels that would have been toxic to *B. napus* indicating that the transfer of the metal hyperaccumulating phenotype is feasible. Somatic hybrids capable of removing significant amounts of Pb were also obtained from *B. juncea* and *T. caerulescens* (Gleba et al., 1999). Collectively, this research illustrates the potential of biotechnology to enhance the effectiveness of phytoremediation.

Metal hyperaccumulators are notorious for small size and slow growth. These properties have an adverse impact on the potential for metal phytoextraction and severely restrict the employment of effective agronomic practices such as, mechanical harvest. To overcome these disadvantages, conventional breeding approaches have been proposed to improve plants for metal extraction. Unfortunately, the success of this approach may be precluded due to sexual incompatibility caused by anatomical differences between parents. Biotechnology has the potential to overcome this limitation by allowing direct gene transfer. Research data indicate that manipulation of relevant plant features, including metal tolerance, via biotechnology is a realistic possibility.

## TECHNOLOGY DEPLOYMENT

Phytoremediation is an emerging technology potentially effective and applicable to a number of different contaminants and site conditions. At this stage, additional experience and information is needed to consolidate phytoextraction into a decision matrix or cost model. Expectations for phytoremediation should also be revised and more appropriately managed. For example, phytoextraction, which is likely to require multiple growing seasons, is a lengthy process compared with traditional engineering approaches. In addition, the potential for cleanup depends upon chemical speciation of the metal, with some chemical species simply unavailable for plant uptake and removal. It should also be added that evaluation of field performance is complicated by the difficulty to characterize the mass balance of metal contaminants. Wide variation of metal concentration (both vertically and horizontally) precludes these efforts. In addition, the problem is compounded by metal leaching away from the original source (e.g.,

following the application of synthetic chelates) and/or alteration of contaminant concentration due to soil tillage.

The cost associated with the implementation of phytoextraction is difficult to estimate because of a lack of economic data. It is likely, however, that the cost will be site specific. Existing data suggest the cost of initial demonstrations may be high (due to monitoring requirements) with a rapid cost decline as we gain more experience and improve the efficiency of the technology.

Recently a group of fundamental and applied scientists with interest in Phytoremediation met to discuss research needs and assess the status of technology deployment (Kuperberg et al., 2000). This group ranked a variety of inorganic contaminants including metals with respect to phytoextraction research status, readiness for commercialization, and regulatory acceptance of the technology. Results of this evaluation are shown in Table 2.

## DISCUSSION

A great deal of evidence indicates that plants have the genetic potential to clean up soil contaminated with toxic metals. Identification of metal hyperaccumulator species has been an impetus for phytoremediation research. Despite significant research efforts, phytoremediation is still an emerging technology. Investigators as well as science administrators have strived to identify and address research needs in an effort to close knowledge gaps and make phytoremediation a reliable cleanup technology. It is possible, however, that in addition to scientific issues this transition would also require a new research approach. Complex interactions that take place under site specific conditions require that metal phytoextraction must be approached as a multi disciplinary research effort. Success will ultimately depend on the employment of a holistic approach to integrate the work of plant biologists, soil microbiologists, agronomists, and environmental engineers. These experts will have to work together focusing on identifying and solving numerous and diverse scientific issues posed by metal phytoextraction. For example, for Pb phytoextraction soil chemists will have to work to enhance Pb bioavailability for uptake while avoiding groundwater contamination. To ensure a research continuum, biologists will have to identify solutions to optimize the plant's ability to take up and store Pb. Agronomists will have to further provide solutions to applied issues such as, how to incorporate amendments including synthetic chelates in soil, and when and how to harvest. Such an integrated approach will allow the formulation of a comprehensive research plan, ensure research continuity, and avoid redundancy.

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