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Improved Understanding of Hyperaccumulation Yields Commercial Phytoextraction and Phytomining Technologies

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This paper reviews progress in phytoextraction of soil elements and illustrates the key role of hyperaccumulator plant species in useful phytoextraction technologies. Much research has focused on elements which are not practically phytoextracted (Pb); on addition of chelating agents which cause unacceptable contaminant leaching and are cost prohibitive; and on plant species which offer no useful phytoextraction capability (e.g., *Brassica juncea* Czern). Nickel phytoextraction by *Alyssum* hyperaccumulator species, which have been developed into a commercial phytomining technology, is discussed in more detail. Nickel is ultimately accumulated in vacuoles of leaf epidermal cells which prevents metal toxicity and provides defense against some insect predators and plant diseases. Constitutive up-regulation of trans-membrane element transporters appears to be the key process that allows these plants to achieve hyperaccumulation. Cadmium phytoextraction is needed for rice soils contaminated by mine wastes and smelter emissions with 100-fold more soil Zn than Cd. Although many plant species can accumulate high levels of Cd in the absence of Zn, when Cd/Zn > 100, only *Thlaspi caerulescens* from southern France has demonstrated the ability to phytoextract useful amounts of Cd. Production of element-enriched biomass with value as ore or fertilizer or improved food (Se) or feed supplement may offset costs of phytoextraction crop production. Transgenic phytoextraction plants have been achieved for Hg, but not for other elements. Although several researchers have been attempting to clone all genes required for effective hyperaccumulation of several elements, success appears years away; such demonstrations will be needed to prove we have identified all necessary processes in hyperaccumulation.

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The focus of this review is phytoextraction, a developing technology that uses plants to accumulate elements from contaminated or mineralized soils and transport the metals to shoots which may be harvested to remove the elements from the field. Phytoextraction is a subset of phytoremediation which includes phytostabilization, phytovolatilization, phytodegradation, and other plant-based technologies to remediate or stabilize contaminants in the environment. Reviews on varied aspects of phytoextraction have appeared from several research groups with each discussing the potential merits of phytoextraction from their unique point of view (Salt et al., 1998; Baker et al., 2000; Chaney et al., 2000, 2005; Lasat, 2002; McGrath et al., 2002; Pollard et al., 2002; Meagher and Heaton, 2005; Pilon-Smits, 2005; Yang et al., 2005a, 2005b).

Natural element hyperaccumulator plant species can be effective in phytomining or phytoextraction of particular elements from contaminated or mineralized soils (e.g., Lasat, 2002; McGrath et al., 2002; Chaney et al., 2005; Banuelos, 2006). The most widely accepted definition of "hyperaccumulator" using Ni as an example is "... a plant in which a nickel concentration of at least 1000 $\mu g\ g^{-1}$ has been recorded in the dry matter of any aboveground tissue in at least one specimen growing in its natural habitat." (Reeves, 1992, p. 261). At the same time, large numbers of papers have appeared which examined concepts of phytoextraction we believe are not useful for contaminated or mineralized soils. These methods are unacceptable due to metal leaching (added chelating agents), or impossible due to selection of plants with no practical ability to selectively extract unusually high levels of metals from a natural soil, or from mixtures of soil elements, as is the case with true hyperaccumulator species.

Phytoextraction depends on the annual accumulation of enough of a given element in harvestable shoot biomass that removal of the biomass would support soil remediation or phytomining goals (Table 1). It was recognition of the potential to remove enough metal in one crop of a hyperaccumulator species to make progress in soil decontamination that inspired Chaney (1983) to suggest the concept of

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Table 1. Example plant species which hyperaccumulate elements to over 1% of their shoot dry matter, usually at least 100-fold levels tolerated by crop species.

Element	Plant species	Maximum metal concentration	Location collected	Reference
		mg kg^{-1} dry wt.		
Zn	*Thlaspi caerulescens*†	39 600	Germany	Reeves and Brooks, 1983b
Cd	*Thlaspi caerulescens*	2908	France	Reeves et al., 2001
Cu‡	*Aeolanthus biformifolius*	13 700	Zaire	Brooks et al., 1978
Ni	*Phyllanthus serpentinus*	38 100	New Caledonia	Kersten et al., 1979
Co‡	*Haumaniastrum robertii*	10 200	Zaire	Brooks et al., 1978
Se	*Astragalus racemosus*	14 900	Wyoming	Beath et al., 1937
Mn	*Alyxia rubricaulis*	11 500	New Caledonia	Brooks et al., 1981
As	*Pteris vittata*	22 300	Florida	Ma et al., 2001
Tl	*Biscutella laevigata*	15 200	France	Anderson et al., 1999

† Ingrouille and Smirnoff (1986) summarize consideration of names for *Thlaspi* species; many species and subspecies were named by collectors over many years (Reeves and Brooks, 1983a, 1983b; Reeves, 1988).

‡ Although Cu and Co hyperaccumulation were confirmed in field collected samples, similar concentrations have not been attained in controlled studies.

phytoextraction. Baker and Brooks (1989) similarly stressed the key role of hyperaccumulator species in the possibility of phytoextraction. As research on phytoextraction has progressed, it has become increasingly apparent that the inherent metal hypertolerance of natural element hyperaccumulators is key to obtaining levels of Ni, Zn, Cd, Se, Co, As, Tl (Table 1), and some other elements in plant shoots high enough to achieve phytoextraction goals. Normal plants simply do not tolerate high enough concentrations of elements in their leaves to allow significant annual removal of soil elements (Table 2) (Chaney et al., 2005), although some have argued that low biomass hyperaccumulators were no better than crop plants in phytoextraction of some elements (Ebbs and Kochian, 1998). Annual biomass yield is important, but the element tolerance and accumulation in shoots are the key traits that must be considered. Thus crop plants are rarely useful for practical phytoextraction (Chaney et al., 2000; McGrath and Zhao, 2003). In general, hyperaccumulators accumulate 100-fold higher shoot metal concentration without yield reduction than do crop plants.

When there exists no known plant species which hyperaccumulates an element, one searches for weeds which accumulate unusually high levels compared to other species. Lasat et al. (1998) found that red root pigweed (*Amaranthus retroflexus* L.) accumulated soil ^{137}Cs to levels which may support a phytoextraction technology, while crop plants were much less able to accumulate Cs. Another weed, the desert shrub "chamisa" or gray rabbitbrush (*Chrysothamnus nauseosus* (Pall.) Britt.), was found to accumulate ^{90}Sr from deep in contaminated soils at the Los Alamos National Lab more effectively than other local plants (Fresquez et al., 1996). Unfortunately, no controlled experiments were conducted with chamisa to identify the selectivity of Sr uptake by this species compared to known crop species which had been characterized for relative Sr and Ca uptake and translocation.

Lead Phytoextraction and Chelator-Induced Phytoextraction

With the large number of articles about the use of Indian mustard (*Brassica juncea* Czern) in phytoextraction of Pb, we believe the development of phytoextraction went astray. We believe the desire to phytoextract Pb from soils seemed to be such a good economic opportunity to some researchers that they tried to develop Pb phytoextraction technologies. It had long been known that if soils have enough phosphate to give good crop yields, most of the Pb remained in the soil or in plant roots (Chaney and Ryan, 1994; Ryan et al., 2004). Only with P deficiency stress is Pb translocated to shoots in significant amounts (Koeppe, 1981). The original Pb phytoextraction work by Kumar et al. (1995) examined Pb uptake from solution cultures with deficient phosphate and sulfate so added Pb remained soluble. Pb was absorbed and killed the plants; some of the dead *B. juncea* plants contained on the order of 10 g Pb kg^{-1} dry weight in shoots.

Brassica juncea had very little ability to absorb Pb from contaminated soils. When Blaylock et al. (1997) and Huang et al. (1997) looked for methods to aid or "induce" Pb phytoextraction from soils, they found that adding EDTA could both desorb soil Pb so it could move to the roots, and the PbEDTA chelate could leak through root membranes and be transported to shoots with transpiration. *B. juncea*, one of the first identified Pb accumulators (Kumar et al., 1995), accumulated high concentrations of Pb when EDTA was applied to soils—much more than average plant species. Part of the success was due to injury of the root membranes by EDTA not chelated to Pb (Vassil et al., 1998). Research was conducted by numerous groups to find a way to make "chelator-induced in situ phytoextraction" effective and safe in the environment, but the added chelating agents caused unavoidable leaching of chelated metals (e.g., Pb) down the soil profile (Römkens et al., 2001; Lombi et al., 2001a; Madrid et al., 2003; Schmidt, 2003; Wenzel et al., 2003b; Wu et al., 2004). In several field tests of EDTA on firing range soils in the US, rapid leaching of Pb to groundwater proved an unacceptable side effect of chelator-induced Pb phytoextraction. Similar risk from leaching of ^{60}Co and other

Table 2. Estimated Ni phytoextraction by corn (*Zea mays* L.) vs. *Alyssum murale* grown as a phytomining crop; assume soil control soil contains 25 mg Ni kg^{-1}, and the Ni-rich soil contains 2500 mg Ni kg^{-1} = 10 000 kg Ni (ha 30 cm)$^{-1}$; assume soil Ni is sufficiently phytoavailable that corn has 50% yield reduction compared to grown on similar soil without Ni mineralization. Research has shown that unimproved Alyssum murale can easily yield 10 t ha^{-1} with fertilizers, and selected cultivars can exceed 20 t ha^{-1} with appropriate soil and crop management with Brockman variant cob. clay loam serpentine soil (5000 mg Ni kg^{-1}) in the field (Li et al., 2003b). Most crop plant species suffer 25% yield reduction when the shoots contain 100 mg Ni kg^{-1} dry weight (Kukier and Chaney, 2004).

Species	Soil	Yield	Ni in the crop			Ash-Ni
		dry t ha^{-1}	mg kg^{-1}	kg ha^{-1}	% of soil	%
Corn	Control	20	1	0.02	0.01	0.002
Corn (50% YD)	Ni-rich	10	100	1	0.01	0.20
Wild *Alyssum murale*	Ni-rich	10	20 000	200	2.0	20–40
Alyssum murale cultivar	Ni-rich	20	25 000	500	5.0	25–50

radionuclides in contaminated soils was a well known effect of EDTA (ethylenediaminetetraacetate) and NTA (nitrilotriacetate) in radionuclide "cleaning solutions" spilled onto contaminated soils (Means et al., 1978), so this unacceptable effect of added chelating agents used for in situ Pb phytoextraction should not have been a surprise. When mass balances were calculated for EDTA-induced Pb phytoextraction, the fraction of Pb which reached the shoots was a very small fraction of the Pb which leached from the topsoil. The characteristic of allowing added EDTA to substantially increase metal chelate translocation to shoots seen in *B. juncea* is unusual. Zn-EDTA transport was much greater for *B. juncea* than three other species tested (Collins et al., 2002).

In addition to the risk of metal leaching, EDTA is an expensive chemical. Little discussion of the potential cost of EDTA-induced phytoextraction occurred, but this issue seriously detracts from that the feasibility of that technology. Chaney et al. (2002) obtained the price of commercial quantities of EDTA and estimated the cost would be about \$30 000 ha^{-1} for the amount of EDTA reportedly needed to attain over 10 g Pb kg^{-1} dry shoots (10 mmol EDTA kg^{-1} soil for each cropping). Chelating agents which are more rapidly degraded by soil microbes yet chelate Pb well (e.g., EDDS) are more expensive and do not appreciably decrease metal leaching from treated soil (Meers et al., 2005).

Many plants have been studied which have no practical ability to accumulate any elements above those of crop plants because they lack the specialized tolerance and accumulation mechanisms of hyperaccumulator plants. Indian mustard is a significant oilseed crop in India, and a tasty root vegetable for hot and sour soup, that has been studied extensively despite it having no evident phytoextraction ability for any element (including Se). Others have tested Cd accumulation by tumbleweed (*Salsola kali*) (De la Rosa et al., 2004), and Pb phytoextraction by vetiver grass (*Vetiveria zizanioides* Nash) (Chen et al., 2000; Boonyapookana et al., 2005), lemon-scented geranium (*Pelargonium crispum*) (KrishnaRaj et al., 2000), sunflower (*Helianthus annuus* L.), and wheat (*Triticum aestivum* L.), etc. Claims that Ni phytoextraction may be useful with white clover (*Trifolium repens* L.) (Yang et al., 1996), fababean (*Vicia faba* L.) (Srivastava et al., 2005), and other species with no unusual ability to tolerate or accumulate Ni from soils have not been substantiated.

These potential problems with soil application of chelators are one family of problems with research methods in phytoextraction. Another aspect of chelators has also caused many studies to be confounded by metal-chelate interactions. It has been known for many years that addition of Zn, Cu, and many other metals to nutrient solutions containing FeEDTA causes displacement and precipitation of the Fe and chelation of part or all of the added test metal ion. Parker et al. (1995) reviewed the use of chelating agents in nutrient solutions, showing how to avoid most of the serious problems, at least for dicots. Chelators which form highly selective chelates with ferric are good Fe sources for dicots, but do not suffer displacement of Fe when other metal ions are added to the solution at the practical pH range of plant experiments. Many papers reported findings which were seriously confounded by precipitation of Fe and chelation of the metal under study.

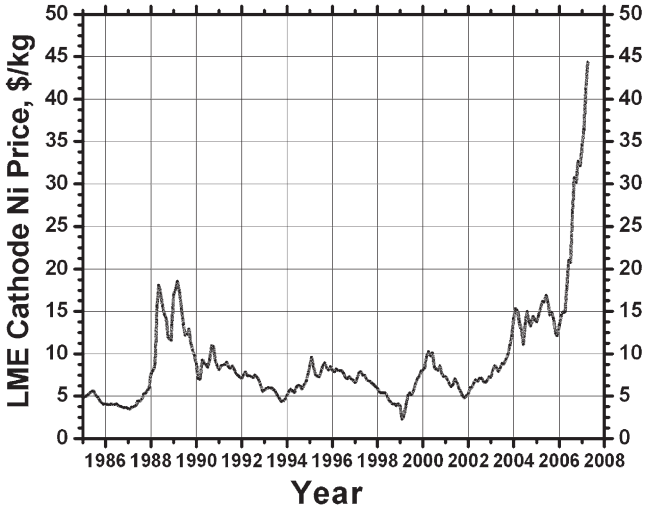

Fig. 1. Price of nickel metal on the London Metal Exchange, 1985 through April 2007 (based on data from U.S. Geological Survey).

Nickel Phytoextraction and Phytomining

Chaney et al. (1998, 2000, 2005) and Li et al. (2003b) showed that *Alyssum murale* Waldst. & Kit. and *Alyssum corsicum* Duby could accumulate higher than 20 000 mg Ni kg^{-1} shoots dry weight with no evidence of phytotoxicity when grown on serpentine soils with minimal addition of fertilizers. Further, with modern use of herbicides and other agricultural management practices, one can grow biomass containing 400 kg Ni ha^{-1} with production costs of \$250 to \$500 ha^{-1}. At the time this article was prepared, Ni metal was trading on the London Metal Exchange at more than \$40 kg^{-1} (Fig. 1), so Ni phytomining has become a highly profitable agricultural technology (crop value = \$16 000 ha^{-1}) for Ni-contaminated or mineralized soils. Li et al. (2003b) reported highly effective Ni recovery from *Alyssum* Ni hyperaccumulator shoot biomass ash in an electric arc furnace at the Inco Ltd. Sudbury smelter complex. Because the bulk of plant ash is nutrient elements that do not interfere with Ni recovery from the ash, *Alyssum* biomass ash is the richest known Ni ore. Considering the low productivity of infertile serpentine soils for agricultural crops and the high value of Ni that can be annually phytomined with normal fertilizer inputs, Ni phytomining should emerge as a profitable agricultural industry (Li et al., 2003b; Chaney et al., 2005). Further, phytomining is much less disruptive of the land than strip mining "lateritic serpentine" deposits.

Benefits of Hyperaccumulation to Plants

The evolution of the hyperaccumulator trait is believed to have occurred because the trait benefited the plant (Boyd and Martens, 1992, 1994; Baker et al., 2000; Meharg, 2003). Research on specific ways plants could benefit has identified a number of chewing insects to which Ni-rich or Zn-rich leaves are toxic. Most, but not all, insects stop consuming the high metal leaves or suffer severely reduced growth rates. Inhibition occurs with as little as 5000 mg kg^{-1}, far below the levels attainable in hyperaccumulators. A review by Coleman et al. (2005) and article by Jhee et al. (2005) reported testing of multiple pests with Ni-rich biomass. Similarly,

Behmer et al. (2005) summarized progress in characterizing the role of high foliar Zn in limiting feeding by insects. Jiang et al. (2005) showed hyperaccumulated Cd protected against feeding damage by thrips.

Another hypothesis for the benefit of metal hyperaccumulation was "elemental allelopathy" (Boyd and Martens, 1992). Boyd and Jaffré (2001) examined limitation of colonization of other plants in the zone beneath *Sebertia accuminata* trees in New Caledonia and found some Ni enrichment in topsoil. Zhang et al. (2007) examined the effect of incorporation of Ni-rich *Alyssum* biomass into both the serpentine soil (Brockman cobbly loam) used to grow the *Alyssum* and a Sassafras sandy loam which has low Ni and little ability to sorb Ni. They found that the biomass Ni was so quickly sorbed in the near neutral serpentine Brockman soil from Oregon that there was no increase in toxicity of soil with added Ni-rich biomass, while for the Sassafras soil, added biomass limited germination of a number of common weeds. Thus for serpentinite-derived soils rich in Fe and Mn oxides and near neutral pH, the decomposition of Ni-rich biomass on the soil near hyperaccumulator plants does not appear to be a significant limiting factor in plant germination.

Value of Phytoextraction Crops

Robinson et al. (1997) considered the value of different elements known to be hyperaccumulated. Several elements may provide economic phytomining potential with known plant species (Ni, Co, Tl, Au), while the value of other elements are unlikely to balance the cost of crop production to remove them from contaminated or mineralized soils (As, Pb, Cd, ^{137}Cs, Cu, Se). Biomass energy value can offset some costs of phytoextraction crop production. Recently increasing values of metals suggests that even Zn recovered ($3.80 kg^{-1}) could contribute to the cost of cleaning up Zn-Cd contaminated soils (assuming 25 000 mg Zn kg^{-1} in healthy shoots).

Biomass energy crops which remove significant amounts of some metals have been studied as an economic phytoextraction technology. If leaves were collected from poplar or willow, which are known to accumulate some Cd, removal of soil Cd could occur over decades of biomass-energy cropping (Granel et al., 2002; Robinson et al., 2003a; Pulford and Watson, 2003). Other elements are not removed rapidly enough to make this a promising technology although boron phytoextraction could solve particular problems (Robinson et al., 2003a). Poplar trees tolerated high foliar B, evapotranspiration prevented dispersal of a B-rich groundwater plume, and the leaves could be marketed as an organic B fertilizer which would reduce the cost of groundwater B remediation at the test site (Robinson et al., 2003a).

Banuelos and Mayland (2000) showed that Se phytoextracted into plant biomass from Se-rich soils could be used as a Se feed supplement. The value in feed was characterized, but market development for ground biomass of a Se accumulator has not progressed. It surely seems wiser to recycle Se from contaminated soils needing remediation than to mine virgin Se ores. In addition, mixing Se-rich biomass into a mixed feed would result in a more uniform mix than with a Se salt. Banuelos (2006) also discussed recovering other value that can make phytoextraction more cost effective. Because high soil sulfate inhibits selenate uptake by crop plants, Banuelos et al. (2003) argues that growing food crops with higher Se levels in growers fields for sale as nutrient-rich foods would be more likely to solve soil Se enrichment problems rather than trying to treat agricultural drainage water. Because Se deficiency is epidemiologically linked with increased risk for certain cancer, Se enrichment of foods is being actively investigated.

Another beneficial use of hyperaccumulator biomass Ni has been identified—fertilizer for Ni-deficient soils. Wood et al. (2005) reported economically significant yield loss and even death of pecan trees (*Carya illinoinensis* (Wangenh.) K. Koch) on low Ni Coastal Plain soils of Georgia, USA. Nickel fertilizers sprayed on the trees cured the deficiency. We tested use of water extracts of Ni-rich *Alyssum* biomass as a replacement for the commercial $NiSO_4$ initially used in field testing and found that the biomass Ni was at least as effective as a Ni salt fertilizer (Wood et al., 2006). *Alyssum* Ni fertilizer could be produced at far lower cost per kg of Ni than $NiSO_4$. *Alyssum* Ni fertilizer could qualify as an "organic" Ni fertilizer and be applied as a water extract by spray or fertigation, or ground *Alyssum* biomass incorporated into surface soil.

Cadmium Phytoextraction

Fruitful progress has been made in phytoextraction of Cd from soils contaminated by geogenic Zn+Cd sources. Unfortunately, as with Pb, much of the reported research has little relevance to practical Cd phytoextraction. Chaney et al. (2005) explain how the Zn/Cd > 100 in soils contaminated by mine wastes and most other sources of Cd and Zn causes Zn hyperaccumulation to reach phytotoxic levels and limit yields of most plant species before much Cd can be accumulated in shoots. Nearly all soils which require Cd remediation to protect human health are rice soils with Zn+Cd contamination (Chaney et al., 2004). Thus any plant used for Cd phytoextraction must tolerate the high Zn which co-occurs with the Cd contamination. Several plant species can accumulate over 100 mg Cd kg^{-1} if Zn is omitted (e.g., *Brassica juncea* [Ebbs et al., 1997]; *Avena strigosa* [Uraguchi et al., 2006]; tumbleweed [*Salsola kali*] [De la Rosa et al., 2005]), but not when soils contain normal Zn/Cd ratios of geogenic sources (200 g Zn/1 g Cd) because Zn usually kills the plants with only about 5 to 10 mg Cd kg^{-1} dry weight. The single exception is *Thlaspi caerulescens* J. & C. Presl. strains from southern France which accumulate about 10-fold higher Cd/Zn ratio in their shoots than is present in the soil. When the shoots contain 20.0 g Zn kg^{-1}, Cd can exceed 2000 mg kg^{-1} dry weight (Li et al., 1996; Lombi et al., 2000; Reeves et al., 2001; Zhao et al., 2002; Basic et al., 2006; Keller et al., 2006; Li et al., 2006). This yields annual Cd removal sufficient to make rapid progress in reducing risk from soil Cd. The optimum pH for phytoextraction may need to be established for each soil (Wang et al., 2006). Actually, *T. caerulescens* can tolerate over 10 000 mg Cd kg^{-1} dry weight when Cd is supplied in a soluble form with minimal Zn (Mádico et al., 1992).

Another approach is breeding cultivars of crop plants which could phytoextract enough Cd to achieve soil remediation. Ae and Arao (2002) and Murakami et al. (2007) tested Cd phy-

toextraction from contaminated soils using relatively high Cd accumulating genotypes of rice grown under upland conditions and observed 20 to 50 mg Cd kg^{-1} DW in shoot biomass. It is evident that Zn phytotoxicity limits rice Cd accumulation and annual removal compared to hyperaccumulator species, although shoot biomass yields are much higher than presently known Cd hyperaccumulators. Genetic variation in Cd accumulation is known in other crop species (Grant et al., 2007), but in the presence of the geogenic Zn/Cd, Cd removals are not sufficient to allow useful soil remediation (e.g., Nehnevajova et al., 2005).

Much of the Cd phytoextraction research focused on increasing phytochelatins (PCs) or metallothioneins in plant shoots was misguided. It was assumed that if more chelators were present in plants, Cd would be less phytotoxic. The apparent induction of PCs by Cd suggested that increasing biosynthesis of phytochelatins would improve tolerance and phytoextraction, but there has been no evidence that this approach would yield a Cd phytoextraction plant. At best, Cd tolerance was increased 3- to 7-fold by high expression of PC synthase (e.g., Heiss et al., 2003). Compared with the 200 times higher tolerance of Zn and Cd of *Thlaspi caerulescens* from southern France (Reeves et al., 2001; Chaney et al., 2005; Wang et al., 2006), this 3- to 7-fold Cd tolerance improvement in crop plants is trivial. Higher PCs or metallothioneins concentrations do not necessarily increase shoot Cd concentration. Similar findings have been observed when researchers tried to alter Cd accumulation in tobacco shoots by transgenic expression of phytochelatin or metallothioneins despite extensive testing of many constructs (Lugon-Moulin et al., 2004).

Phytochelatins are a family of sulfhydryl-rich compounds whose biosynthesis involves a glutathione precursor with additions of cysteine-γ-glutamate. When plants are fed phytotoxic levels of Cd, glutathione is drained into Cd chelation and replacement is biosynthesized. An enzyme (phytochelatin synthase) can synthesize phytochelatins if the products are depleted by chelation, but the induction by metals is an artifact of severe metal toxicity, not a mechanism whereby plants prevent phytotoxicity (Vatamaniuk et al., 2000; Schat et al., 2002). Millions of dollars have been spent on the study of phytochelatin biochemistry although environmental studies showed that these compounds played no important role in metal tolerance (Schat et al., 2002), or hyperaccumulation of soil metals (Ebbs et al., 2002). An article by Maier et al. (2003) reported a detectable increase in lettuce shoot phytochelatin when lettuce leaves contained 100 mg Cd kg^{-1} DW, about 25 times higher than acceptable in foods. The fraction of shoot Cd bound by shoot phytochelatins was small (mole ratio < 0.25 for whole shoots considering 2 moles phytochelatin sulfhydryl per mole Cd). Presently the hypothesis that improving phytochelatin synthesis would aid in development of plants for practical phytoextraction is unsupported.

Although some have hypothesized modifying plants to secrete a metal-specific chelating agent that can mobilize soil-bound metals and be absorbed by the roots in the manner of phytosiderophores (Raskin, 1996), there is no evidence that such an approach could be developed into practical phytoextraction systems. Phytosiderophores are relatively non-specific chelators; Ni can displace Fe from the Fe-deoxymugineic acid chelate and induce Fe deficiency (Kukier and Chaney, 2004). Although the concept of making plants secrete chelating ligands into the rhizosphere and then absorb the metal-ligand complex into roots remains plausible for phytoextraction, it should not be considered a method likely to be developed. One needs a biosynthetic compound actively secreted by roots that can mobilize select soil-bound metals in the rhizosphere and then be absorbed by roots and transported to the plant shoots.

Phytoavailability in the Rhizosphere

Nutrient (or metal) acquisition from soil is a rate-limiting step in phytoextraction and a challenge for plant nutrition. Root-induced processes linked with nutrient acquisition occur in the rhizosphere of all higher plants and include fluctuations in pH and redox potential, secretion of enzymes, root exudation, and perturbation of equilibria between solid and solution. In general, these rhizosphere processes act to increase the phytoavailability of elements in the root zone above those levels in bulk soil. Naturally, one might hypothesize one or more of these processes were adapted by hyperaccumulator plants to achieve hyperaccumulation; however, it is clear these processes are not unique to hyperaccumulators whereas constitutive up-regulation of trans-membrane element transporters is a key trait for hyperaccumulation (Pence et al., 2000; Assunção et al., 2001; Bernard et al., 2004). Therefore, root-induced changes in the rhizosphere play only an indirect role in element acquisition through their influence on the lability of various elemental species in soil adjacent to roots (i.e., rhizosphere effect on phytoavailability).

Interestingly, both Cd and Ni hyperaccumulator plants sample the same labile pool of soil element as normal crop plants. It is not selective accumulation of occluded soil metal by the hyperaccumulator species that allows hyperaccumulation (Echevarria et al., 1998; Gerard et al., 2000; Hutchinson et al., 2000; Shallari et al., 2001; Schwartz et al., 2003; Hammer et al., 2006).

Soil pH affects the solubility of trace elements (cations become more soluble at more acidic pH, while anions become more soluble at higher pH, due to sorption on soil solid phases), and hence would be expected to affect the phytoavailability of soil metals to hyperaccumulator plants. Brown et al. (1994) found that *Thlaspi caerulescens* accumulated Zn and Cd more effectively at more acidic pH, in agreement with the solubility of Zn and Cd in the test soils. Wang et al. (2006) observed that phytotoxicity of soil Zn and Al could limit yield of *Thlaspi caerulescens*, and that maximum annual Cd removal may need to be identified for soils where commercial phytoextraction would be conducted. Interestingly, *A. murale* and *A. corsicum* followed the opposite and unexpected pattern for effect of pH on Ni solubility and hyperaccumulation (Li et al., 2003a; Kukier et al., 2004). For soils with a few percent Fe oxides, increasing soil pH increased Ni hyperaccumulation even though it reduced the solubility of soil Ni. For high Fe serpentine soils, raising pH toward 7 appreciably reduced Ni hyperaccumulation, presumably because the sorption of Ni would be stronger in soils with higher amorphous Fe oxide levels. A subsequent test of the effect of nutrient solution pH on hyperaccumulation of Ni confirmed that *Alyssum corsicum* accumulated much higher levels of Ni with increasing solution pH (Peters et al., 2000), indicating the effect was

a property of the plant rather than the soil. It is likely that the form of the transmembrane Ni^{2+} transporter which is most active in Ni uptake is favored at pH ~7.5 rather than lower pH.

Researchers have attempted to identify ligands which were believed to be secreted by roots of hyperaccumulators to increase the rate of Ni release from soil and/or uptake by roots (Salt et al., 2000; Krämer et al., 2000; Pinel et al., 2003; Puschenreiter et al., 2003; Wenzel et al., 2003a, 2003b). An important test compared hyperaccumulator *Thlaspi* species with wheat which secretes phytosiderophores. The wheat rhizosphere solution contained substantial levels of ligand(s) while the hyperaccumulator rhizosphere solutions contained very little (Zhao et al., 2001). Other research looked for changes in pH, redox, or other activities in the rhizosphere which might be altered by hyperaccumulators to allow them to phytoextract massive amounts of metals; but comparison of closely related species subjected to the same treatments showed no unique ability of hyperaccumulators to acidify the rhizosphere, to secrete amino or organic acids, or to reduce Fe (Gabbrielli et al., 1991; Bernal and McGrath, 1994; Krämer et al., 2000; Pinel et al., 2003). Most evidence supports the model in which up-regulation or constitutive high activity of element transporters in plasma membranes, (e.g., plasma membranes of root epidermal cells, xylem parenchyma cells, cells in the leaves, and tonoplasts of leaf cells) which allow plants to achieve hyperaccumulation (as opposed to using secreted ligands). While ligand secretion and other common root processes do not allow plants to achieve hyperaccumulation, they can alter elemental speciation in the rhizosphere and thereby influence the phytoavailability of elements in the rhizosphere.

Interestingly, rhizosphere microbes may increase Ni and Zn hyperaccumulation from soils (Delorme et al., 2001; Whiting et al., 2001; Lodewyckx et al., 2002; Idris et al., 2004; Abou-Shanab et al., 2003, 2006) although hyperaccumulation can occur in sterile soils. The mechanism whereby inoculation of a non-sterile serpentine soil with an organism from the rhizosphere of a hyperaccumulator can significantly increase metal accumulation by species such as *Alyssum murale* and *Thlaspi caerulescens* remains unknown.

Two groups have examined the fundamental soil-plant relationship in Zn phytoextraction by *T. caerulescens*, testing solute transfer models for Zn uptake by several species (Whiting et al., 2003; Sterckeman et al., 2004). These models are more general and estimate processes in the soil and root rather than molecular phenomena. This testing indicated that diffusion from the soil was more rate-limiting than convection of Zn to the root with transpiration. *T. caerulescens* has especially fine and long roots, providing a high root surface area for metal absorption. High rate uptake occurs at considerably higher Zn concentration than present in most cropland. Several authors have reported that *T. caerulescens* has a very high requirement for both the concentration of Zn required in leaves for normal functions, and the Zn concentration in soil solution needed to attain maximum Zn uptake rate (Li et al., 1995; Ozturk et al., 2003).

The portion of root length which participates in high rate Zn and Cd uptake and transport to shoots has not been reported. It is likely that only young root tips (1–3 cm behind tip) have a high rate of uptake and translocation of Zn as has been reported for other polyvalent cations by normal crops (Harrison-Murray and Clarkson, 1973). This relationship needs to be characterized for hyperaccumulator species. Whiting et al. (2000) also found that roots of *T. caerulescens* displayed a zincophilic growth pattern—roots were more concentrated in volumes of soil which were richer in phytoavailable Zn. This may be related to the higher Zn requirement of this species compared to normal crops, but the causation remains a topic of research.

Lombi et al. (2002) tested the hypothesis that the IRT1 protein shown to play the key role in uptake and transport of Fe^{2+} by plants was involved with the exceptional Cd hyperaccumulation of *T. caerulescens* from southern France. They showed a correlation between Fe supply to roots and short-term Cd uptake-translocation to shoots. On the other hand, Cohen et al. (1998, 2004) tested whether the IRT1 protein was involved with uptake of Cd and some other elements. The earlier study (Cohen et al., 1998) compared high concentrations of the test elements and found IRT1 could transport several elements. However, their later research (Cohen et al., 2004) tested concentrations of metals found in soil solution, and concluded that IRT1 was important only in Fe^{2+} uptake. We remain unconvinced that the *T. caerulescens* from southern France are highly effective in Cd hyperaccumulation in the presence of Zn because of a mutation in their IRT1 protein. Further, Cosio et al. (2005) evaluated Cd and Zn accumulation by leaf slices and found a large difference in Cd accumulation by leaf slices of 'Ganges' compared to 'Prayon' types of *Thlaspi*, indicating that the substantial genetic difference in Cd accumulation could be seen at the leaf cell level. This may contradict the interpretation of Lombi et al. (2002) regarding the large difference in Cd accumulation by *T. caerulescens* genotypes.

Selenium Phytoextraction

Selenium mineralized soils and drainage water evaporation ponds in California and several other U.S. states cause an environmental Se hazard. Original concern about excessive Se focused on poisoning of grazing livestock which consume Se hyperaccumulators when other forages are not available. In the evaporation ponds, however, aquatic food chains gave high biomagnification of Se; fish and birds experienced Se toxicity (Ohlendorf et al., 1986). Phytoextraction is seen as an inexpensive alternative method to alleviate risk from Se-rich soils.

High selectivity in uptake of selenate vs. sulfate (selective selenate accumulation), coupled with effective metabolic Se detoxification in plant tissues (high Se tolerance), appear to be the key traits for effective Se phytoextraction and soil remediation. In nearly every case of soils with excessive Se, high levels of sulfate are also present. Sulfate inhibits selenate uptake by crop plants, but the Se hyperaccumulators effectively accumulate Se in shoot biomass (as high as 1% Se in shoot dry wt.) from soils rich in sulfate and selenate. Bell et al. (1992) examined the effect of sulfate concentration on Se accumulation by alfalfa (*Medicago sativa* L.) compared to *Astragalus bisulcatus* [Hook.] Gray) and found that *Astragalus* continued to hyperaccumulate Se when sulfate was increased whereas Se levels in alfalfa declined sharply with increasing sulfate.

Banuelos et al. (2003) noted the potential to grow Se-enriched

crop plants to remove the Se from the irrigated fields rather than attempting to deal with the more concentrated mixture of sulfate, borate, and selenate in drainage waters. The cost of Se phytoextraction from these natural Se-rich soils could be offset from sales of the biomass as Se-enriched foods or as a Se feed supplement (Banuelos and Mayland, 2000). Parker et al. (2003) and Goodson et al. (2003) have evaluated several hyperaccumulator and non-hyperaccumulator species for ability to accumulate Se from contaminated or mineralized soils. The difficulty in germination and low yields of *Astragalus* led them to evaluate another well known Se accumulator species, *Stanylea pinnata* (Pursh) Britton; they found high shoot Se, much easier germination, and larger yield than for *Astragalus* species. Both Se hyperaccumulator species accumulated Se in the presence of high levels of soil sulfate, confirming the selective uptake of selenate needed for practical phytoextraction (Feist and Parker, 2001; Parker et al., 2003).

The transgenic approach to higher Se accumulation was attempted by Pilon-Smits et al. (1999) who overexpressed ATP sulfurylase in *Brassica juncea*. They found increased uptake and tolerance of Se in the transgenic plants, but the uptake selectivity between selenate and sulfate was not improved. Neither Se accumulation nor Se tolerance approached those of the natural Se hyperaccumulators. Again, transgenic expression of several proteins in crop plants or *Arabidopsis* gave small significant increases in Se tolerance, but little ability to phytoextract Se from real contaminated soils (Ellis et al., 2004).

Another approach was tested by LeDuc and Terry (2005) using soil microbes and plants to phytovolatilize dimethylselenide or to reduce selenate to inorganic Se^0 which precipitates in wetland soils, and into organic-Se compounds in soil organic matter residues. This was a drainage or effluent water treatment technology, and the soil medium would need to be removed and replaced when soil Se^0 and organic Se accumulate to higher levels to maintain the treatment effectiveness.

Development of Phytoextraction Technologies

Two approaches for the development of commercial phytoextraction technologies are believed to have significant promise: (i) domesticate natural element hyperaccumulators; (ii) clone all genes needed for hyperaccumulation and hypertolerance and express them in a high-biomass yielding transgenic hyperaccumulator. Several reviews of developing transgenic plants to achieve phytoextraction have appeared (Clemens et al., 2002; Cherian and Oliveira, 2005; Pilon-Smits, 2005), but these articles did not consider the limitations of phytochelatins and Pb accumulation discussed in the present review. The domestication of natural hyperaccumulators has already been shown to be successful for Ni, Cd, Se, and As phytoextraction (Chaney et al., 2005; Meagher and Heaton, 2005). In the transgenic approach, only Hg has been demonstrated to work in the field (Heaton et al., 2003) but public acceptance has been difficult because Hg^0 is volatilized at the soil surface and will eventually be re-deposited on soil or water.

The Hg phytovolatilization development is an example of gene transfer that was successful after creative application of science. Initially the Hg reductase was cloned from bacteria and was poorly expressed in plants; but when the DNA sequence was modified, it was highly expressed in several plant species. Then the organic Hg lyase was cloned, modified to obtain higher expression in plants, and the improved plant tested (Heaton et al., 2003). With poor acceptance of Hg volatilization technologies, the research team has worked to build a plant which collects Hg in the shoot biomass (Meagher and Heaton, 2005). Most plants have little translocation of Hg through the xylem; most shoot Hg is collected from gaseous Hg which is emitted from the soil surface (e.g., Gustin et al., 2004). To date, Meagher and coworkers have evaluated expression of phytochelatins to increase Hg and As binding in plants to construct a plant which can retain Hg and an Angiosperm which hyperaccumulates As.

One genomic approach to understanding hyperaccumulators examined *Thlaspi caerulescens* cDNAs expressed in yeast which increased yeast tolerance to Ni. These studies showed that nicotianamine synthase (NIS) expression increased Ni tolerance in yeast (Vacchina et al., 2003), and coupled with inductively coupled plasma mass spectrometry identified the presence of nicotianamine in the cells (Schaumloffel et al., 2003). Expression of NIS in *A. thaliana* conferred nicotianamine accumulation and resistance to Ni (Pianelli et al., 2005). These researchers also identified a nicotianamine-Ni-Fe transporter (*TCYSL3*) in *Thlaspi caerulescens* 'Ganges' (Gendre et al., 2007). Mari et al. (2006) noted that Ni-nicotianamine accumulated in the roots of Ni-exposed *Thlaspi caerulescens*, but the enzyme was only expressed in the shoot. Thus nicotianamine is involved with Ni circulation in *Thlaspi* (Mari et al., 2006). One should keep in mind that Ni-nicotianamine may accumulate in root cells because Ni becomes a sink for nicotianamine by chelating with Ni^{2+}. The specific role of nicotianamine in Ni tolerance, transport, and hyperaccumulation remains unsettled. As noted earlier, a very small fraction of the Ni in the latex formed by the extreme Ni hyperaccumulator *Sebertia accuminata* was present as Ni-nicotianamine (Schaumloffel et al., 2003). This finding of a very small role for nicotianamine in binding Ni in an extreme Ni hyperaccumulator illustrates a weakness in the strategy of cloning metal tolerance genes of higher plants in yeast. Although yeast may allow identification of a gene with the potential to increase Ni binding, this observation alone does not demonstrate a role in the normal physiology of hyperaccumulator plants.

A recent examination of genes expressed in *Thlaspi caerulescens* vs. *A. thaliana* using the microarray devices based on genes expressed in *A. thaliana* provides important advances in understanding of the unusual characteristics of *T. caerulescens*. Van de Mortel et al. (2006) found large differences in expression of genes for Fe and Zn homeostasis between *A. thaliana* and *T. caerulescens*. Rigola et al. (2006) found that 4289 expressed sequence tags (ESTs) were generated from Zn exposed root and shoot tissue. By comparison with *A. thaliana*, they found that 8% of the ESTs had no significant similarity with known genes and appear to be specific to *T. caerulescens*. The *T. caerulescens* transcriptome generally related well to that of *A. thaliana*, although a relatively large number of *T. caerulescens*-specific transcripts were found. In addition, *T. caerulescens* expressed a relatively large number of genes which are expressed at a much lower level in *A. thaliana*. Thus there is need for clarification of the regulation of different

genes involved in metal tolerance and metal hyperaccumulation, including full characterization of promoter sequences associated with the key genes. Genomic, transcriptomic, and proteomic approaches can each contribute to improved understanding of phytoextraction biochemistry and agronomy.

Although much research funding has been invested in characterizing and cloning genes used by natural plants to achieve hyperaccumulation, no complete transgenic hyperaccumulator is near field testing. Additionally, questions are raised about the high biomass yielding crop plant that should be selected to host the hyperaccumulator genes to develop a transgenic hyperaccumulator for commercial phytoextraction (Angle and Linacre, 2005). Further discussion of concerns about transgenic plants which can accumulate potentially toxic levels of elements can be found in Watrud et al. (2006). Perhaps pollen-free sterile transgenic plants can alleviate concerns about invasive toxic weeds, or transfer through pollen of high element accumulation into crop plants, but this would add considerable cost and time for development of transgenic phytoextraction cultivars needed for known soil contamination problems.

There is general agreement that as fundamental understanding of the mechanisms used by hyperaccumulator plants is improved, both approaches to development of commercial phytoextraction technologies will be aided. Very rapid and selective uptake of metals, rapid transport to shoots, and very effective storage of metals in leaf cell vacuoles appear to provide the mechanisms for hyperaccumulation. For example, Küpper et al. (1999) developed a novel method to extract vacuolar sap from frozen leaf cells of *Thlaspi caerulescens*; they found Zn was 385 m*M* in the sap, a remarkable bioconcentration by these cells.

High root uptake of Zn and Cd by *Thlaspi caerulescens* was found to be constitutive; that is, uptake was not down-regulated by high supply of Zn to the plants (Pence et al., 2000; Assunção et al., 2001, 2006; Lombi et al., 2001b). For normal plants, when Zn accumulates in root cells the root cell membrane Zn transporters become down-regulated and uptake rate is reduced substantially. This lack of down-regulation of Cd uptake is confirmed in the study of *T. caerulescens* grown on Cd-amended, Zn-deficient soil with varied additions of Zn fertilizer (Ozturk et al., 2003).

Plants appear to use two general mechanisms to protect cells from hyperaccumulated elements. During translocation, metals appear to be chelated with organic acids (malate, citrate), or amino acids (histidine, nicotianamine); after the metals reach shoots they are stored in vacuoles or in/on cell walls. The Ni and Zn hyperaccumulators have unusually high basal levels of malate and citrate and these organic acids may increase with increasing shoot Ni or Zn. Krämer et al. (1996) reported that histidine was about 80% as high as Ni in xylem exudate of *Alyssum lesbiacum*. But Ni forms both mono- and di-histidine chelates, so the fraction of Ni chelated by histidine is likely lower than 80%; alternatively, mixed Ni-histidine-malate complexes may occur in the xylem exudate rather than the Ni(his) chelate. Organic acids or inorganic anions such as sulfate clearly supplied adequate complexation capacity for the Ni. Localization of Zn and Cd in leaves of *Thlaspi caerulescens* was found to be predominantly in vacuoles of large epidermal cells, but some of the Cd remained in the extracellular space within the leaf (Küpper et al., 1999; Frey et al., 2000; Cosio et al., 2005). Some Zn and Cd also remained in the apoplast. Ma et al. (2005) found little evidence for Cd localization in the apoplast; and although the concentration of Cd and Zn in epidermal cells was about double that of mesophyll cells, because there more mesophyll cells, these cells held double the amount of Cd and Zn as the epidermal cells. And in contrast with some early reports, there was no association of vacuolar Zn or Cd with Cl, P, or S. Interestingly, Zn and Cd were not accumulated in the guard cells and subsidiary cells of the stomatal complex. It is not yet clear whether a vacuolar influx pump would achieve this pattern of metal ion distributions among leaves, but highly selective expression of transporter proteins will be needed to achieve these results. An elegant new study using ^{113}Cd nuclear magnetic resonance (NMR) examined the association of Cd with malate or citrate in *T. caerulescens* (Ueno et al., 2005). It is clear that Cd was associated with malate in this species, including in intact leaves. Whether Ni remains associated with malate in vacuoles of hyperaccumulators has not been demonstrated yet.

Persans et al. (1999) attempted to improve the Ni accumulation or tolerance of *Thlaspi* species by adding genes for each enzyme which they believed could increase biosynthesis of histidine. Additional plant histidine had little effect on Ni tolerance and no useful effect on Ni hyperaccumulation. Additional histidine did not make a normal plant into a hyperaccumulator.

More recently, Freeman et al. (2004) found that increasing biosynthesis of the metabolite (O-acetyl-L-serine) needed to make glutathione improved metal tolerance and appeared to be part of the Ni tolerance mechanism of *Thlaspi goesingense* Halacsy even though there is little evidence that Ni is normally chelated with cysteine or glutathione. Lee et al. (1977) found many of the New Caledonian Ni hyperaccumulators contained Ni-citrate, but that *Alyssum* species and some others had more Ni-malate than Ni-citrate in water extracts of leaves of field-grown plants. Kersten et al. (1980) showed that extracts of leaves *Psychotria douarrei* had 63% of Ni as Ni-malate, while *Phyllanthus serpentinus* had 42% of Ni as Ni-citrate and 40% as Ni-malate; they confirmed the negatively charged Ni chelates by electrophoresis. In the liquid latex formed by the Ni hyperaccumulator tree *Sebertia acuminata*, Schaumloffel et al. (2003) found that 0.3% of the Ni was present as the chelate with nicotianamine and 99.4% as Ni-citrate. Therefore chelation with organic acids and transport to xylem and vacuoles are normal mechanisms hyperaccumulator plants use to protect their cellular biochemistry from free metal ions.

Very interesting studies of intracellular localization of elements have been reported for Ni and Zn: these elements are stored in the vacuoles of epidermal and adjacent mesophyll cells in leaves, thus preventing accumulated metals from disrupting normal metabolic processes in the cells (Küpper et al., 1999, 2001, 2004; Broadhurst et al., 2004a). Further studies have shown that only the vacuole in the base of trichome cells contains Ni, not the trichome proper (Broadhurst et al., 2004b). Although Ni was initially reported to have been accumulated in trichomes on leaves of *A. lesbiacum* (Krämer et al., 1997), it is now clear that this was an artifact of using colorimetric reagents and treatments which released Ni from the storage vacuoles. When cells are kept frozen, or examined in

vivo with X-ray imaging, it is clear that vacuoles are loaded with Ni, whereas the Ca-rich non-glandular trichomes have only low Ni levels if any (Psaras et al., 2000; Robinson et al., 2003b; Broadhurst et al., 2004a, 2004b; McNear et al., 2005; Tappero et al., 2007).

In a study of both Ni and Co uptake and storage by *Alyssum murale*, Tappero et al. (2007) found that although both Ni and Co were absorbed by roots and translocated to shoots, Ni was compartmentalized in epidermal cells while Co remained in the transport system (extracellular space and cell walls) and was preferentially localized near leaf tips and margins. Additionally Co was partially secreted from leaves and formed a sparingly-soluble phase on leaf surfaces (exocellular sequestration). X-ray tomography images of Co and Ni in hydrated *A. murale* leaves revealed that Co was not stored in leaf epidermal cells, suggesting the specialized biochemical processes linked to Ni hypertolerance do not confer hypertolerance to the co-accumulated Co (Fig. 2).

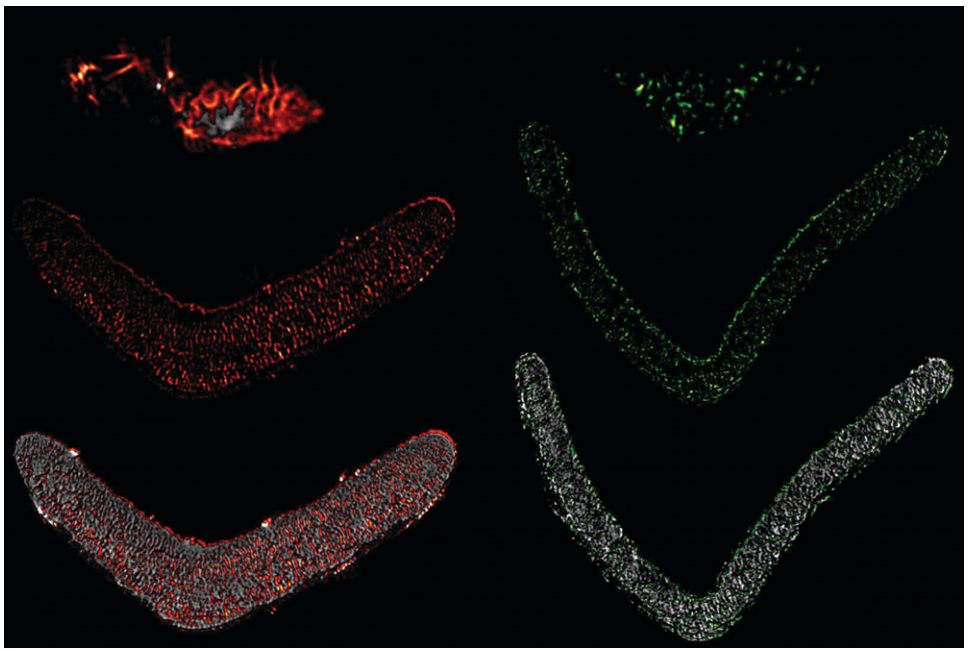

Fig. 2. X-ray tomography images of Co (red) and Ni (green) in hydrated *Alyssum murale* leaves showing the distribution of metal in the leaf tip (top) and bulk leaf (middle) regions and metal distribution in relation to the leaf cell structure (bottom). Images were acquired with synchrotron-based differential absorption computed-microtomography.

Genetic-Physiological Studies with *Arabidopsis halleri* L.

Arabidopsis halleri L. (syn. *Cardiminopsis halleri*) is a Zn hyperaccumulator that is very small and hyperaccumulates only Zn from geogenic metal-rich soils (this species is tolerant but does not hyperaccumulate Cd and Ni as found for *T. caerulescens*). Because *A. halleri* is closer to *A. thaliana*, several researchers have conducted important genetic studies with this species (Bert et al., 2000). Zinc tolerance and hyperaccumulation have been found to be constitutive in *A. halleri*. Thus, genetic tests within the species cannot evaluate the number of genes involved. Macnair et al. (1999) made crosses between *A. halleri* and *Arabidopsis lyrata* L. var. *petraea* L. and backcrosses to allow evaluation of the inheritance. They found that Zn hyperaccumulation and Zn tolerance were separate genetic properties in *A. halleri*. Zinc tolerance appeared to be controlled by a single gene, while Zn and Cd hyperaccumulation appear to be dependent on several genes (Macnair et al., 1999; Bert et al., 2003). Evaluation of differences between *A. halleri* accessions has found small variation in metal accumulation, but no apparent difference in inheritance of metal accumulation (Bert et al., 2000).

There is general acceptance of the idea that study of the processes used by model plants to accomplish different metabolic goals is the most promising path to fuller understanding. *Arabidopsis thaliana* is being used as the model dicot in diverse studies but it is not a hyperaccumulator. Peer et al. (2006) have been undertaking basic studies to identify a true hyperaccumulator species close to *Arabidopsis* that could be used in metabolic and gene expression studies of hyperaccumulators. Peer et al. (2006), Assunção et al. (2003), and Chaney et al. (2005) concluded that *T. caerulescens* from southern France offers great promise in understanding effective hyperaccumulation of multiple metals, and the population from St. Félix de Pallières is especially promising. Salt and colleagues have nominated the southern France ecotype of *T. caerulescens* for sequence determination in the U.S. Department of Energy sequencing program (personal communication, 2007).

The progress in identification of QTLs (quantitative trait loci) associated with hyperaccumulation, and cloning of genes related to hyperaccumulating show the promise of modern biological technologies in understanding physiology and biochemistry of such processes. The ultimate proof that a gene plays a significant role in a process is production of a knockout mutant. This is nicely illustrated by the original report of Eide et al. (1996) who found the *IRT1* gene for ferrous uptake in *A. thaliana*, and the subsequent report of Vert et al. (2002) who characterized the knockout mutant. The knockout lacked only the uptake of Fe^{2+}. All other physiological processes known to be related to Fe uptake remained effective. But the knockout *IRT1* plants were extremely susceptible to chlorosis, indicating that knocking out the one gene stopped essentially all Fe uptake. We can only hope that in the next few years knockout mutants of some of the genes believed to be involved in Zn, Cd, Ni, and other element hyperaccumulation will provide the ultimate clarification of which genes are key to hyperaccumulation. The constitutive up-regulation of expression of the root Zn transport protein of *Thlaspi caerulescens* (Pence et al., 2000) also shows the need to ultimately characterize the regulation of hyperaccumulation with full understanding of the promoter regions of the relevant genes.

Most researchers have assumed they need to identify every

activity of hyperaccumulators required to achieve hyperaccumulation. Alternatively, one could transfer to hyperaccumulators genes which increase plant height, biomass, and/or growth rate or otherwise improve adaptation to contaminated soils (e.g., adaptation of *Thlaspi* to Cd-contaminated rice soils).

Recovery of Metals from Phytoextraction Biomass

As noted previously, the value of Ni in phytomining biomass can exceed \$16000 ha^{-1}, and methods to recover and market this metal are needed. Li et al. (2003b) reported Ni metal was readily recovered from the ash of *Alyssum* biomass when 500 kg of ash was placed in a "revert bag," then added to an electric arc furnace at the Inco Ltd. smelter complex (Sudbury, Ontario, Canada). The Ni was very readily recovered from the ash. Indeed, biomass ash is the richest Ni ore offered to date. Most Ni ores are rich in silicates and/or Fe and Mn oxides, both of which inhibit recovery of Ni from the ores, but biomass ash contains mostly water-soluble plant nutrients that do not interfere with Ni recovery.

In the case of Cd and Zn, greater volatility requires stronger emission controls, but offers the opportunity to separate these metals from the remaining biomass ash components by fractional distillation. This premise was tested by Ljung and Nordin (1997) and appears to offer reasonable processing for phytoextraction biomass (Keller et al., 2005). Others have tested pyrolysis of either biomass plus metal salts (Koppolu and Clements. 2003) or hairy root cultures which accumulated metals (Boominathan et al., 2004). It is unlikely that biomass plus metal salts can model plant biomass rich in metals; further, root cultures rich in metals don't model plant shoots either. In contrast with other suggestions (Sas-Nowosielska et al., 2004), it seems clear that biomass which has no ore value can be efficiently disposed in landfills or hazardous waste landfills perhaps with volume reduction by composting, anaerobic digestion, or other processes. Only in the case of radionuclides does it seem clear that the contaminants in biomass will require expensive disposal after successful phytoextraction. Facilities for incineration of biomass containing radionuclides are limited, and processing this biomass would require substantial hauling costs.

Agronomy of Phytoextraction

Annual phytoextraction depends on both the bioaccumulation ratio (plant/soil metal concentrations on dry matter basis) and the harvestable yield. In the end, the agronomy of producing high yields of phytoextraction crops is central to success of this technology. Several groups have conducted large field tests of phytoextraction and these illustrate some of the constraints on successful technologies (Li et al., 2003b; Keller et al., 2003; McGrath et al., 2006).

The *Alyssum* Ni phytomining technology was developed for commercial phytomining (Chaney et al., 2005). The overall development program (Li et al., 2003b) has been described. One must characterize fertilization and pH optima, plant density effects on shoot Ni yield, and develop improved cultivars. Li et al. (2003b) show the range of genetic variation in shoot Ni concentration they observed for over 150 collected genotypes of *A. murale* and *A. corsicum*. Both were tall (1 m at flowering) and could be harvested with normal farm equipment; after brief field drying, the biomass could be baled for shipping to a biomass burn facility. Scheduling of harvest has to be based on avoiding defoliation during flowering and seed filling; because most of the Ni is in the leaves, it is important to harvest the shoots before much defoliation occurs. This has the additional benefit of limiting viable seed dispersal. These Ni hyperaccumulator *Alyssum* species were self-incompatible, so recurrent selection had to be used to breed improved strains rather than simple crossing. Selection was based on both yield, retention of leaves into flowering, shape of plants, and Ni concentration in the harvestable yield. The best strains they tested on the Brockman cobbly loam contained 2.7% Ni dry wt. of aboveground biomass.

Fertilizer requirements were estimated from both pot and field experiments. *Alyssum murale* has remarkable ability to obtain soil P, which likely evolved due to the low P and high Fe status of most serpentine soils. Although L'Huillier et al. (1996) reported they had to supply over 1000 kg P ha^{-1} to a New Caledonia serpentine soil to obtain full yield of maize (*Zea mays* L.), Li et al. (2003a) found that 100 kg P ha^{-1} to serpentine soils gave full yield of *A. murale*. Higher N fertilization increased Ni accumulation and yield; split N application is best to limit excessive N application while obtaining maximum annual biomass Ni yields. Serpentine soils are also very low in Ca and high in Mg, but *A. murale* accumulates Ca so well that no yield response to Ca was observed in our tests. However, it is clear that repeated harvest and removal of the biomass in phytomining would require Ca fertilization. With continued cropping, normal fertilization requirements of crops would have to receive attention as well.

Soil pH management for *Alyssum* phytomining was characterized by both pot experiments and field testing. In early pot tests (Li et al., 2003a), lowering pH increased uptake of Zn, Mn, and Co, but reduced uptake of Ni; and raising pH reduced uptake of Zn, Mn, and Co but increased Ni concentration in shoots. Thus even though raising pH reduces the concentration of Ni in soil solution, it increased shoot Ni concentration and yield of Ni in shoots. Additional studies to characterize this response were reported by Kukier et al. (2004). Field testing of liming vs. sulfur addition found increased shoot Ni and yield with limestone addition to the Brockman cobbly loam, and reduction in yield and shoot Ni with sulfur addition. In the pot experiments it appeared that the high concentration of Fe oxides in serpentine soils cause an increased sorption of Ni as pH is raised, so liming of the Port Colborne regional non-serpentine soils gave a large increase in shoot Ni, whereas field liming gave a smaller increase in shoot Ni on the Oregon serpentine soils compared to no increase in the pot tests.

Another important agronomic management practice is weed control. When serpentine soils are fertilized to produce maximum *Alyssum* biomass, other plants can compete. Many serpentine soils are in Mediterranean climate regions so that water availability limits yield, making weed control even more important to limit water use. Many of the herbicides useful with *Brassica* crops are compatible with *Alyssum*, and preplant incorporation of herbicides aided in establishment of plant stands. No perfect herbicide is available to control dicot weeds in *Alyssum* fields.

One more agronomic issue became evident in field demonstrations near a Ni refinery in Port Colborne, Ontario, Can-

ada. In that location soils are frozen in winter and very wet due to poor drainage. In the third year of field testing at Port Colborne, heavy rainfall in early spring caused field flooding and *Alyssum* became infected and many plants died before the normal harvest date. In the fourth year of field testing the plants were established with ridge tilling, and effective surface drainage at the end of ridges. In this way the adverse effect of wet spring weather did not reduce *Alyssum* yields.

Significant field testing of *Thlaspi caerulescens* was conducted by Keller et al. (2003), Keller and Hammer (2004), and Hammer and Keller (2003) in Switzerland, and is now being conducted by Simmons, Angle, and cooperators in Thailand (personal communication, 2007). Research has shown the low P requirement for a full yield of biomass, and that N fertilization increases the yield of Cd and Zn in shoots. Because this species grows so slowly from seed, most tests have used transplants of 4- to 6-wk-old seedlings. In addition, the plants are short, seldom above 30 cm, and have a rosette growth pattern which makes harvest difficult. Although the plants are perennial, and repeated harvests can be made by hand, no mechanical harvest has been demonstrated which would not kill the plants. In the case of *Thlaspi*, Zn and Cd hyperaccumulation are increased with reduction in soil pH as noted earlier, and the low pH needed to optimize annual phytoextraction helps in limiting competition from weeds by Zn phytotoxicity. With the herbicides approved for *Brassica* crops, one can readily control the grass weeds common to high Zn soils. Harvest techniques are needed to make Cd phytoextraction more cost effective.

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