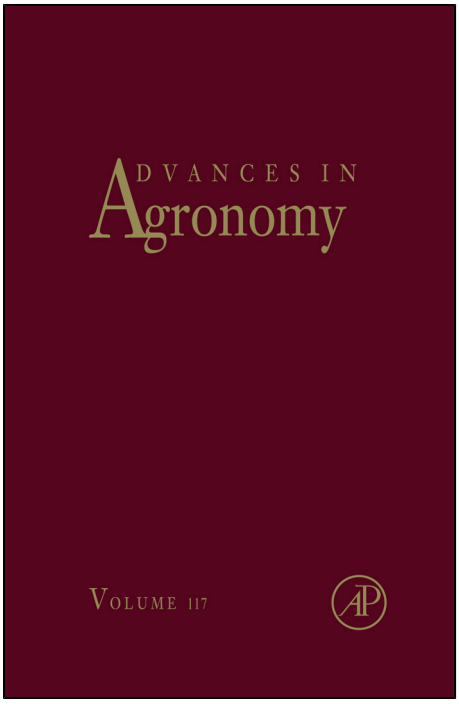

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CHAPTER THREE

Mechanisms of Nickel Uptake and Hyperaccumulation by Plants and Implications for Soil Remediation

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Abstract

Soil contamination by heavy metals like Ni was originally restricted to metalliferous soils but in modern time it has become a general problem due to increasing anthropogenic activities. Because of the characteristics of cost-effectiveness, environmental friendliness, and fewer side-effects, the development of plant based remediation technologies for the cleanup of Ni-contaminated soils has attracted much attention. Nickel is an essential micronutrient, but is toxic to plants at excessive levels. Some plant species can accumulate Ni in the shoots at a high concentration, these plants are called hyperaccumulators. In the past two decades, researchers have endeavored to understand the physiological and molecular mechanisms of Ni uptake, transport, and detoxification in the Ni-hyperaccumulator plants. This is the basis of creating ideal plants for phytoremediation through cell and genetic engineering technologies, which may subsequently improve phytoremediation efficiency for decontaminating Ni-contaminated soils. Both rhizosphere microorganisms and endophytes can play a role in phytoremediation. Optimizing plant and soil management practices, particularly the correction of soil pH and additions of amendments of exogenous chelates and fertilizers, can also enhance phytoremediation of Ni-contaminated soils. The primary objective of this review is to discuss the recent progresses in basic and applied research relevant to phytoremediation of Ni-contaminated soils.

1. Introduction

Nickel (Ni), the 24th element in the order of natural abundance in the Earth's crust (Garrett, 2000), is an essential mineral nutrient for plants in natural soils (Marschner, 1995). However, the natural geochemical processes such as weathering of ultramafic rocks, and increasingly frequent anthropogenic activities, including industrial operations (mining, smelting, electroplating, steeling) and products (alloy, motor vehicles, aircraft paint, chemical, textile, pigment), agricultural utilization of fertilizers and pesticides, waste combustion and sewage sludge spreading, have dramatically increased soil Ni contamination (Gimeno-García *et al.*, 1996; Kopittke *et al.*, 2007; Rajkumar and Freitas, 2008b). The increasingly reported Ni contamination of soil has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects on both wildlife and human beings through food chains (Guo and Marschner, 1995; Salt *et al.*, 1995a). Therefore, increasing emphasis has been placed on the remediation of Ni polluted soils, and several technologies have been developed in recent years for this purpose.

Current technologies for cleaning contaminated soils such as physical (e.g., mechanical/pyrometallurgical separation and absorbent fixation) and chemical remediation (e.g., soil amendment and chelate-enhanced

leaching) are extensively used. These methods are efficient and even endemically commercialized, but they are prohibitively expensive, labor intensive, soil disturbing, and usually have potential environmental and ecological risks (Mulligan *et al.*, 2001; Krämer, 2005; Wu *et al.*, 2010). More recently, phytoremediation, the use of live plants to remove excess heavy metals from soil, has emerged as a promising alternative. Phytoremediation, which can be accomplished *in situ*, is relatively cost-effective, environmentally friendly, and the soil can be utilized simultaneously or immediately after treatment application (EPA, 1998; Ensley, 2000). The benefits of phytoremediation have been demonstrated in many heavy-metal contaminated sites, therefore this technology has been utilized by many environmental companies (Glass, 2000).

Phytoremediation includes several subsets such as, phytoextraction, phytostabilization, rhizofiltration, and phytovolatilization (Raskin and Ensley, 2000). In rhizofiltration, plant roots absorb, concentrate and/or precipitate toxic metals from polluted effluents (Dushenkov *et al.*, 1995). In phytovolatilization, limited extent plants extract volatile metals such as Hg and the non-metal Se from soil and volatilize them from foliage (Bañuelos, 2000; Zayed *et al.*, 2000). Phytostabilization and phytoextraction are the two main strategies in soil phytoremediation (Salt *et al.*, 1998; Huang and Chen, 2003). The former aims at the stabilization of the metals usually in the moderately to heavily contaminated soils by rendering them harmless, thus preventing their migration to surrounding ecosystems by wind and water erosion (Bãnuelos *et al.*, 1997; Burken and Schnoor, 1999). Phytoextraction is the most effective, but also technically the most difficult phytoremediation strategy (Nedelkoska and Doran, 2000; Chaney *et al.*, 2007). Phytoextraction uses metal-accumulating plants to transport and concentrate metals from soils to the above-ground shoots. By harvesting and post-treatment of the Ni-accumulated biomass, a "bio-ore" may be produced from the Ni-contaminated soils; this process is referred to as "phytomining". Considering the purification feasibility and commercial value of Ni, this method is a commercially acceptable and environmentally sound approach (Brooks, 1998; Chaney *et al.*, 2007; Li *et al.*, 2003b; Sas-Nowosielska *et al.*, 2004).

Usually, if a plant can accumulate Ni more than 1000 mg kg^{-1} dry weight (DW) (0.1%) in shoots, it is defined as a Ni hyperaccumulator (Brooks *et al.*, 1977). Currently, more than 450 hyperaccumulating plant species from 45 families, accounting for less than 0.2% of all known plant species, have been described (Kotrba *et al.*, 2009). The most numerous metal-accumulating plants are the Ni hyperaccumulators. However, no plants identified in wild collections combine all the properties of an ideal hyperaccumulating plant. The ideal plant species for commercial phytoextraction of Ni from polluted or Ni-rich soil should grow rapidly, have high biomass superior capacity for Ni tolerance, and accumulate

a significantly higher Ni concentration than present in the soil (Chaney *et al.*, 2000; Lasat, 2002; McGrath *et al.*, 2002). Therefore, researchers have dedicated themselves to a thorough understanding of the mechanisms of Ni uptake and hyperaccumulation by plants at the physiological, biochemical, molecular, genetic, and agronomic levels. Some approaches such as utilization of microorganisms, genetic engineering, and cell engineering, as well as soil and plant management have been applied to enhance the effectiveness of phytoremediation for the Ni-contaminated soils.

In this review no attempt has been made to cover all the available literatures on uptake and hyperaccumulation of Ni in plants. Several excellent articles on this topic are available: Marschner, 1995; Robinson, 1997; Sheoran *et al.*, 2009; Glick 2010; Krämer, 2010; Maestri *et al.*, 2010; Mench, *et al.*, 2010; Ma *et al.*, 2011; Rascio and Navari-Izzo, 2011; Singh *et al.*, 2011.

2. Status of Nickel in Soils

Nickel has typical concentrations of 1–450 mg kg^{-1} in most natural soils (Kabata-Pendias and Pendias, 1992; Bai *et al.*, 2006). However, in polluted soils, Ni concentrations may reach 20- to 30-fold (200–26,000 mg kg^{-1}) higher than the overall range (10–1000 mg kg^{-1}) (Izosimova, 2005). Serpentine soils developed on ultramafic rocks naturally contain high concentrations of Ni, usually in the range 0.1–3% Ni (Brooks, 1987; Kabata-Pendias and Pendias, 1992), averaging 1% Ni (Prasad, 2005). Even though area under serpentine terrain occupies less than 1% of the earth's total exposed surface, it is abundant in ophiolite belts along tectonic plate margins but widely scattered throughout the world and usually supports a unique flora (Morrison *et al.*, 2009). In Albania (Balkans), serpentine soils cover 30% of the land area (Shallari *et al.*, 1998; Bani *et al.*, 2007). Serpentine soils near Pogradec in Albania contain approximately 3000 mg kg^{-1} Ni (Barbaroux *et al.*, 2009). In a smelter in Burrel, 35 km NE of Tirana (Albania), Ni background concentration in those serpentinite-derived soils is approximately 2356 mg kg^{-1} (Shtiza *et al.*, 2005). Serpentinites are relatively common along the western coast of North America (Oze *et al.*, 2004). In California and Oregon there are over 400,000 ha of serpentine soils, where Ni is usually present at the concentrations between 1000 and 7000 mg kg^{-1} (Alexander, 1994; Nicks and Chambers, 1998). Recently, a regional-scale study (Morrison *et al.*, 2009) suggested that the chemically and mineralogically distinctive serpentine rocks outcrop extensively in the Coast Range and Sierra Nevada of California and surface soil samples derived from serpentine rocks of those areas contained 1300–3900 mg kg^{-1} Ni.

Mining and smelting activities are the major sources of heavy metals entering into the environment and can lead to ecological damages (Wei *et al.*, 2009a). The high organic muck soils present in the Port Colborne, Ontario, Canada are ideal for vegetable production. However, emissions from a refinery have left the nearby farmland with elevated Ni concentrations. Extremely high soil Ni concentrations have been detrimental to the agricultural industry in this region and have rendered the neighboring farmland unsuitable for growing fruits and vegetables (McLaughlin, 2002). Frank *et al.* (1982) reported that the concentration of Ni in the 0–5 cm layer of untilled muck soil downwind from the Port Colborne refinery ranged from 800 to >6000 mg kg^{-1}. Moreover, Everhart *et al.* (2006) showed that total Ni concentrations in the muck soils near this refinery at a depth of 0–15 cm ranged from 63 to >22000 mg kg^{-1}. Due to the shortage of irrigation water, industrial and domestic wastewater has been used to irrigate or partially irrigate croplands. Jinchang city of Gansu province is a famous "Nickel City" in China. The mine and surrounding environment has been polluted and destroyed due to the exploitation of metal mineral resources for more than 40 years (Cong, 1997; Zhao, 2000). The total Ni contents of samples from the agricultural soils (0–15 cm in depth) in this area ranged from 139 to 1099 mg kg^{-1} with the mean value of 300 mg kg^{-1}. All of the tested soil samples exceeded the Ni limit of grade II soil environmental quality standards in China (GB15168-1995), and partially exceeded grade III soil environmental quality standards in China (GB15168-1995), indicating severe pollution of the soils in the vicinity of the mine tailings (Wang *et al.*, 2009). In the United Kingdom (UK), agricultural soils that had received sewage sludge contained Ni concentrations as high as 385 mg kg^{-1}, while the background level of Ni in the UK is around 25 mg kg^{-1}, and the maximum allowable in pasture soils set by the European Community is 75 mg kg^{-1} (McGrath, 1995). In an extensive survey of heavy metal concentrations in US agricultural soils, Holmgren *et al.* (1993) reported a maximum Ni concentration of 390 mg kg^{-1} and a mean Ni concentration of 25 mg kg^{-1}.

3. Nickel Effects on Plants

3.1. Nickel Essentiality

Although discharge of Ni is known to have adverse effects on the environment, the essentiality of Ni is now generally acknowledged, based on the numerous symptoms caused by Ni deficiency and its physiological functions and critical roles in various enzymes in plants (Brown *et al.*, 1987;

Fageria *et al.*, 2002; Muyssen *et al.*, 2004). Nickel is currently considered to be a plant micronutrient and is found in the vegetative organs of most plants in the range of 1–10 mg kg^{-1} DW (Assunção *et al.*, 2003). Its deficiency can cause yield reductions. It has been shown in several plant species that low Ni concentrations have promotional effect on plant growth and development (Seregin and Kozhevnikowa, 2006). The available information on optimal and deficient concentrations of Ni is limited, but its role in the urease and hydrogenase metabolism has been used as criteria for critical concentration level (Harish *et al.*, 2008). In different plant species the deficiency levels for Ni^{2+} ranged from 10^{-12} M to 2×10^{-6} M (Muyssen *et al.*, 2004). Nickel is found in several enzymes including urease, hydrogenase, carbon monoxide dehydrogenase, glyoxalase I, peptide deformylase, acetyl-S-coenzyme A synthase, methylcoenzyme M reductase and Ni-containing superoxide dismutase (NiSOD) (Ermler *et al.*, 1998; Mulrooney and Hausinger, 2003). Gerendás and Sattelmacher (1999) showed that Ni addition markedly enhanced the dry matter production of urea-grown plants, but its deficiency significantly reduced the urease activity in the leaves and roots of plants. Nitrogen-fixing microbes require Ni for processing hydrogen gas generated during N_2 fixation (Evans and Sorger, 1966; Taiz and Zeiger, 1998). Low levels of Ni supply may limit the symbiotic hydrogenase activity of *Rhizobium leguminosarum* (Ureta *et al.*, 2005) and consequently influenced the symbiotic N_2 fixation directly (Zobiole *et al.*, 2010). It is known that Ni is incorporated into metallocenters through the coordination with surrounding ligands such as histidine (His) and cysteine (Cys) and for the complete metallocenter assembly (Hausinger, 1990; Hausinger *et al.*, 1996).

Hyperaccumulated metal (such as Ni) concentrations in plant tissues may function as "elemental defense" against some natural enemies, such as pathogens and herbivores (Boyd, 2004). Recently, some studies have confirmed the defensive effects of Ni. For instance, Ni salts were effective as fungicides against leaf and stem rusts on wheat (Graham and Webb, 1991). When *Alyssum murale* and *Alyssum serpyllifolium* ssp. *lusitanicum* are grown in nutrient solution with low Ni concentrations, plants are more susceptible to infection with *Pythium ultimum* (Ghaderian *et al.*, 2000). Alyssum species grown with higher Ni concentrations were protected from fungal attack. Jhee *et al.* (2006) suggested that Ni hyperaccumulation by *Streptanthus polygaloides* effectively protected against the folivore *Plutella xylostella*. Boyd *et al.* (2002) reported that in choice experiments, for *Senecio coronatus*, Ni-hyperaccumulator leaves were less damaged by snails than non-hyperaccumulator leaves. However, the lowest concentration needed to produce defensive effects is unclear. Coleman *et al.* (2005) suggested that Ni concentrations in plant tissues far lower than the minimum hyperaccumulator level can have defensive

benefits for plants. This phenomenon is under further investigation. Considering the different Ni concentration in the populations of the same plant species and even the organs of the same population, Boyd *et al.* (2008) pointed out an interesting assumption: if Ni does confer protection, then leaves of hyperaccumulator *S. coronatus* are much better defended than roots, as leaves contained at least 5-fold more Ni. Similarly, leaves and roots of hyperaccumulator populations are also better defended by Ni than those of non-hyperaccumulator populations. Nevertheless, the defensive effects have been analyzed mostly in laboratory conditions and have considered only one or a few selected herbivores, rather than being tested in the field where hyperaccumulators have to face an array of natural enemies (Boyd, 2007).

3.2. Nickel Toxicity

An excess of a heavy metal is toxic to most plants (Foy *et al.*, 1978; Seregin and Kozhevnikowa, 2006). It has been more than two decades since the significance of Ni as a pollutant was recognized (Iljin, 1991). The most obvious symptoms of Ni toxicity reported in plants are the inhibition of growth, chlorosis, necrosis, and wilting (Eskew *et al.*, 1984; Marschner, 1995; Pandey and Sharma, 2002; Gajewska *et al.*, 2006; Kopittke *et al.*, 2007; Ghasemi and Ghaderian, 2009). Inhibitory effect of toxic levels of Ni on plant growth has been reported by many authors (Pandey and Sharma, 2002; Gajewska *et al.*, 2006; Gajewska and Skłodowska, 2007, 2010). High concentrations of Ni may contribute to the deficiency of nutrients or the divalent cations that can compete with Ni. Nickel stress has been shown to cause a substantial decrease in all macro- and micronutrients in leaves and achenes of sunflower (*Helianthus annuus* L.) and a marked reduction in root and shoot fresh biomass. In this study, higher Ni levels decreased the concentrations of Ca, Mn, and Fe in achenes and N, K, Zn, Mn, and Cu decreased consistently with increasing level of Ni (Ahmad *et al.*, 2011). In nutrient solution, with increasing Ni concentration from 1 to 100 μM, the uptake of Zn, Cu, Fe, and Mn in barley shoot decreased significantly (Rahman *et al.*, 2005). Maize grown in calcareous soil with Ni application decreased P concentration (Karimian, 1995). The concentration of Cl^- and NO_3^- decreased significantly with increasing Ni concentration in barley shoot (Brown *et al.*, 1990).

L'Huiller *et al.* (1996) found that at the Ni concentration of 60 mM, maize root growth was reduced by directly inhibiting root mitotic activity. Nickel alters G-actin conformation, which can affect dynamics of actin filament (AF) polymerization/depolymerization (Dalledonne *et al.*, 1999). Moreover, Ni is a strong mitotic inhibitor of meristematic cells of *Allium cepa* root (Arrunategui-Jimenez *et al.*, 1999). When the

green alga *Spirogyra decimina* was exposed to Ni, its orientation of cortical microtubules was changed from a transverse to a skewed or longitudinal direction (Přibyl *et al.*, 2008). Imbalances of Ni in plants may cause alteration of fundamental metabolic processes (e.g., photosynthesis and transport of photoassimilates from leaves) (Seregin and Kozhevnikowa, 2006), and thus inhibit the growth. In seedlings exposed to Ni, reduced root sink activity as well as the inhibited starch hydrolysis and/or transport of sucrose may result in the photoassimilate accumulation in leaves (Rautio, 2000; Roitto *et al.*, 2005) and thus the root/shoot ratio of Scot pine (*Pinus sylvestris*) was reduced (Rautio, 2000). In addition, accumulation of carbohydrates in shoots was suggested to inhibit the root growth of maize (20−500 mM Ni in the growth medium) (Baccouch *et al.*, 1998). Ni-induced transient starch accumulation was also observed in bundle sheet cells of maize (L'Huillier *et al.*, 1996), needles of pine (Kukkola and Huttunen, 1998), detached rooting bean leaves (Nyitrai *et al.*, 2004), and cabbage leaves (Molas, 2002). The Ni-induced starch accumulation indicates that the export of carbohydrates out of the plastids decreased, most probably due to the lower demand of the rest of the cells as a result of the Ni-dependent inhibition of growth (Appenroth *et al.*, 2010).

Symptoms of Ni toxicity such as chlorosis and necrotic spots often occur in plants. For instance, maize (*Zea mays* L.) leaves became chlorotic at low Ni concentrations (20−100 mM) and necrotic at high Ni (250−500 mM Ni) exposure levels and chlorophyll content decreased (Baccouch *et al.*, 1998). Such chlorosis could result from both Fe and Mg deficiency and the inhibition of chlorophyll synthesis (Seregin and Kozhevnikowa, 2006). The content of photosynthetic pigments chlorophylls a and b decreased considerably after application of Ni (Pandey and Sharma, 2002; Gajewska *et al.*, 2006; Seregin and Kozhevnikowa, 2006; Appenroth *et al.*, 2010). The diminished rate of photosynthesis under Ni stress was reported to relate to the reduced stomatal conductance (Gs) (Bazzaz *et al.*, 1974; Ouzounidou *et al.*, 2006). Maximum quantum yield of primary photochemistry, variable fluorescence (Fv) and chlorophyll concentration were reduced by Ni treatment (Ouzounidou *et al.*, 2006). On the other hand, Ni accumulation may result in acceleration of respiratory rates and disturbance of plant water balance. High uptake of Ni induced a decline in water content of dicot and monocot plant species. The decrease in water uptake has been used as an indicator of the progression of Ni toxicity in plants (Pandey and Sharma, 2002; Gajewska *et al.*, 2006). In addition, several authors have reported that plants responded to Ni exposure can lead to the proline accumulation (Pandey and Sharma, 2002; Kovacik *et al.*, 2009; Gajewska and Skłodowska, 2009; Kazemi *et al.*, 2010). Proline accumulation may be involved in the mechanisms of osmoregulation, as well as, the important

role of proline in response of plants to heavy metal toxicity may be related to its antioxidative properties (Matysik *et al.*, 2002).

At cellular and molecular levels, Ni is known to bind strongly to oxygen (O), nitrogen (N), and sulfur (S) atoms. High affinity of Ni to sulfhydryl groups and disulfide bonds may cause damage to the secondary structure of proteins and affect the enzyme activities, leading to the disturbance of various metabolic pathways (Siedlecka and Krupa, 2002; Kabała *et al.*, 2008). Past studies have shown the effects of Ni on N metabolism: nitrate reductase (NR) and glutamine synthetase (GS) activities and nitrate content (Kevrešan *et al.*, 1998), transaminase activities and amino acid content (El-Shintinawy and El-Ansary, 2000), and proline metabolism (Lin and Kao, 2007). Effect of Ni on the activities of enzymes that mediate the successive steps of N assimilation, such as NR and NiR taking part in nitrate assimilation, GS and GOGAT constituting the main ammonium assimilating cycle, GDH participating in the alternative ammonium assimilation pathway, as well as AlaAT, AspAT catalyzing transamination reactions (Gajewska and Skłodowska, 2009).

Stimulation of production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$ and H_2O_2 indirectly by inhibition of photosynthesis due to high concentrations of Ni in plants have been reported (Gajewska and Skłodowska, 2007; Kumar *et al.*, 2007; Rocchetta and Kuepper, 2009). High Ni induces oxidative stress as evidenced by peroxidation of membrane lipids (Baccouch *et al.*, 1998, 2001). However, the results regarding the effect of Ni on the activities of antioxidative enzymes are inconsistent; both stimulation (Baccouch *et al.*, 1998; Yan *et al.*, 2008; Duman and Ozturk, 2010; Gajewska and Skłodowska, 2010; Israr *et al.*, 2011) and inhibition (Boominathan and Doran, 2002; Pandey and Sharma, 2002) in their activities have been reported. An increase in H_2O_2 content in response to Ni stress was detected in leaves of wheat (Gajewska and Skłodowska, 2007) and canola plants (Kazemi *et al.*, 2010) and in hairy roots of Alyssum (Boominathan and Doran, 2002). H_2O_2 may play an important role in the inhibition of growth of Ni-stressed plants (Chen *et al.*, 2000; Kazemi *et al.*, 2010). The lipid peroxidation destabilizes membranes thus disturbing their structure and function, changing their permeability and affecting the membrane-bound enzyme activities by changing their substrate affinity, activation energy or turnover number (Kerkeb *et al.*, 2001). Nickel affected the lipid composition and H-ATPase activity of the plasma membrane as reported in *Oryza sativa* shoots (Ros *et al.*, 1992a). Generally, the level of malondialdehyde (MDA), an indicator of oxidative membrane degradation, increased drastically in different plants exposed to Ni (Baccouch *et al.*, 1998; Madhava Rao and Sresty, 2000; Ali *et al.*, 2003; Kazemi *et al.*, 2010). Such changes might disturb membrane functionality and ion balance in the cytoplasm, particularly of K^+, the most mobile ion across plant cell

membrane. However, it was reported that Ni did not increase the lipid peroxidation in the isolated tonoplast fractions from cucumber roots (Kabała and Janicka-Russak, 2011). Lipid peroxides content showed no significant changes in wheat leaves after exposure to 100 μM of Ni for 3 days (Gajewska and Skłodowska, 2007). Lack of Ni effect may be related to low metal concentration and/or time of exposure to Ni (Kabała and Janicka-Russak, 2011), however, stimulated lipid peroxidation was reported in plants exposed to Ni at and above 0.5 mM Ni concentrations (Madhava Rao and Sresty, 2000) or application of Ni for several days at micromolar concentrations (Baccouch *et al.*, 1998). An enhancement of membrane lipid peroxidation occurred in *Z. mays* shoots, after 48 h of 250 μM Ni applications (Baccouch *et al.*, 1998). Moreover, the effect of Ni seems to depend on plant tolerance levels (Kabała and Janicka-Russak, 2011). The growth of hyperaccumulator *Thlaspi* on 125 mM Ni for 8 days did not change the total lipid peroxidation, but resulted in a significant increase in MDA levels in non-accumulator *Arabidopsis thaliana* plant (Freeman *et al*, 2004).

Ni-stressed canola plants have shown a decrease in the antioxidant enzymes (CAT, GPX and APX) activities (Kazemi *et al.*, 2010). These enzymes contain Fe in their structure. Since high concentrations of Ni have decreased Fe content in plant tissues (Pandey and Sharma, 2002; Everhart *et al.*, 2006), reduction in their activities in plants subjected to excess Ni may result in the deficiency of Fe for biosynthesis of these enzyme molecules (Kazemi *et al.*, 2010). Thus, the ratio of Ni/Fe may be a good predictor of Ni toxicity in plants.

4. Nickel-Hyperaccumulator Plants

Nickel hyperaccumulator plants contain >1000 mg Ni kg^{-1} dry weight (DW) (0.1%) in the shoots. This is an exceptionally high heavy metal concentration since Ni toxicity in most plants occurs at concentrations higher than 10–50 mg kg^{-1} DW (Marschner, 1995). Excluder plants, stored Ni in root cell wall vacuoles, thus keeping Ni sequestered away from photosynthetically active shoot tissues. Rascio and Navari-Izzo (2011) stated that hyperaccumulating plants actively take up exceedingly large amounts of one or more heavy metals from the growth medium and translocate to the shoots when accumulated at concentrations 100–1000 times higher than those found in nonhyperaccumulating plant species. Hyperaccumulators have a much greater ability to detoxify and sequester huge amounts of heavy metals in the leaves, and exhibit no phytotoxicity symptoms (Rascio and Navari-Izzo, 2011).

Approximately 350 taxa of Ni hyperaccumulators are known to accumulate between 1000 and 38,000 mg Ni kg^{-1} in dry leaf biomass (Reeves, 1992), whereas the corresponding concentrations are below 1–200 mg Ni kg^{-1} for majority of non-accumulator taxa occuring in the same habitats (Brooks, 1987; Wycisk *et al.*, 2004). The web site (http://en.wikipedia.org/wiki/Hyperaccumulators_table_–_2_:_Nickel) lists many of the Ni-hyperaccumulator plant species and their accumulation ability. Additional reports continuously appear on plant species that hyperaccumulate Ni and many yet unidentified Ni-hyperaccumulator plants may exist in other parts of the world (Harish *et al.*, 2008; Rascio and Navari-Izzo, 2011).

Nickel is the heavy metal that has been shown to reach the highest concentration in a plant. This is clearly shown in *Sebertia acuminata* Pierre ex Baill. *Sapotaceae*, a tree which is endemic to the serpentine soil from New Caledonia, can accumulate up to 26% Ni (dry mass) in its latex (Jaffré *et al.*, 1976). A single tree of this species may contain 37 kg of the metal (Sagner *et al.*, 1998). About 25% of discovered Ni hyperaccumulators belong to the family of *Brassicaceae* (Prasad, 2005), in particular, to genera *Alyssum* and *Thlaspi*, and the genera *Alyssum* has the largest number of Ni hyperaccumulators since it hosts more than 50 taxa of such plants (Brooks, 1998; Rascio and Navari-Izzo, 2011). Plants belonging to these species offer an excellent source to study mechanisms of plant adaptation to hostile environments and to evaluate naturally selected complex traits. Linking contrasted phenotypes to specific genetic differences is a first step toward in depth understanding of how these various traits may have evolved (Weigel and Nordborg, 2005; Mitchell-Olds *et al.*, 2007). Such knowledge could then be further used to develop improved biofortification and phytoremediation technologies (Hanikenne and Nouet, 2011).

Most hyperaccumulators are endemic to metalliferous soils behaving as "strict metallophytes" (Rascio and Navari-Izzo, 2011). Ni hyperaccumulation is likely evolved on relatively abundant geogenically Ni-rich (or serpentine) outcrops (Krämer, 2010). A large number of Ni hyperaccumulators may reflect the high worldwide occurrence of serpentine soils. Some "facultative metallophytes" can also live on non-metalliferous soils, although they are more prevalent on metal-enriched habitats (Assunção *et al.*, 2003). Furthermore, there are species that include both metallicolous- and non-metallicolous populations (Rascio and Navari-Izzo, 2011). The plant species that hyperaccumulate Ni are distributed in a wide range of distantly related families and reasons for their evolution that gave rise to hyperaccumulating plants are unknown and still under considerable debate (Hanikenne and Nouet, 2011; Rascio and Navari-Izzo, 2011). For the last 30 years, intensive research has been conducted to understand the biochemical, physiological and genetic basis of hyperaccumulation. Maestri *et al.* (2010) stated that

considering the wide distribution of hyperaccumulators throughout the plant kingdom, it is unlikely that all these species have the same mechanism of hyperaccumulation.

5. Mechanisms of Nickel Hyperaccumulation by Plants

As shown in Fig. 1, metal hyperaccumulation is a complex and rare phenomenon, and the elucidation of the mechanisms for this process constitutes a considerable task. Knowledge of mechanisms of Ni uptake and accumulation from the soil/plant interface, transport to shoot, partitioning and distribution in cellular compartments can lead to an improved understanding of a plant's ability to hyperaccumulate Ni (Everhart *et al.*, 2006; Sheoran *et al.*, 2009).

Maestri *et al.* (2010) concluded that many kinds of transporter proteins participate in metal transport and homeostasis: (i) plasma membrane transporters involved in uptake; (ii) tonoplast transporters for uptake; (iii)

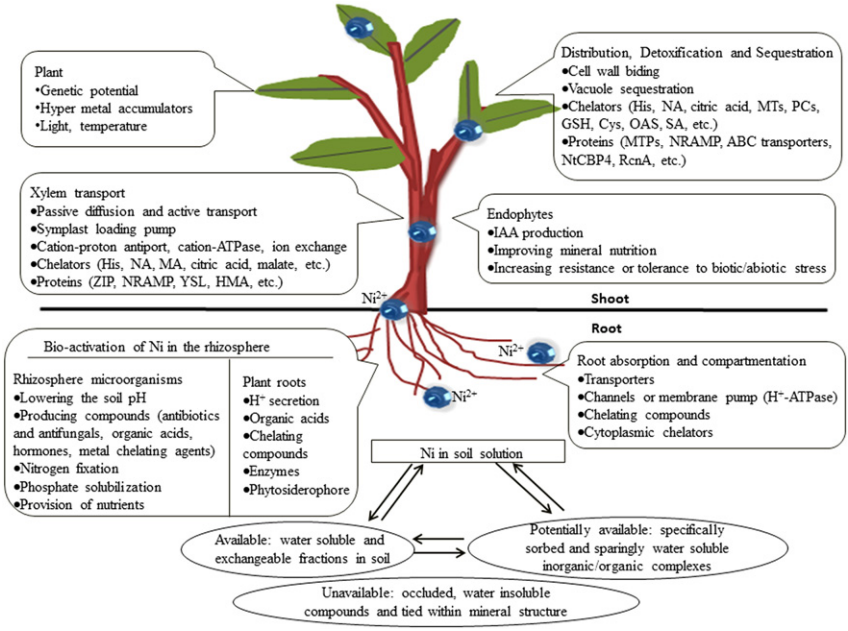

Figure 1 Soil, plant and microbial traits and processes involved in Ni uptake and hyperaccumulation. (Modified from Yang *et al.*, 2005; Sheoran *et al.*, 2009 and Tack, 2010). For color version of this figure, the reader is referred to the online version of this book.

remobilization from the vacuole; (iv) transporters for xylem loading; and (v) endomembrane transporters. Hall and Williams (2003) summarized that some of these families have been identified, such as: (i) the influx transporter families: the natural resistance-associated macrophage protein (NRAMP), zinc-iron permease (ZIP) and yellow-stripe 1-like (YSL) subfamily proteins; (ii) the efflux protein families: the heavy metal ATPase (HMA), the cation diffusion facilitator (CDF) family proteins, cation exchanger (CAX) and ATP-binding cassette transporters (ABC).

5.1. Root Uptake

Plant can directly take up Ni^{2+}, ion pairs (such as $Ni^{2+}(H_2O)_5SO_4^{2-}$), and simple complexes (such as $NiOH^+$) in soil solution (Kabata-Pendias and Pendias, 1992; Tack, 2010). Nickel readily forms complexes with organic substances such as organic acids and other dissolved organic matter, which enhances Ni desorption or dissolution in soils. According to water solubility and/or plant uptake, soil Ni can be divided into available, potentially available, and unavailable pools (Fig. 1). The available Ni pool includes water soluble and exchangeable forms and the potentially available pool constitutes specifically adsorbed Ni, sparingly soluble minerals, and strongly bonded organic complexes, whereas Ni forms occluded in oxides, water insoluble compounds and tied within mineral structure are not available to plants within the growing season and generally considered as unavailable pool (Robinson, 1997; Sheoran *et al.*, 2009). Plants may absorb Ni from available pool at any time. In potentially available pool, Ni is available to plants once the available pool is depleted (Robinson, 1997). The availability of soil Ni is influenced by many factors such as soil pH, redox potentials, organic matter content, and temperature (Kabata-Pendias and Pendias, 1992; Fageria *et al.*, 2002; Tack, 2010).

Much less information is available on Ni uptake, as compared with other micronutrients. Similar to other metal ions, plant uptake of Ni^{2+} may involve both passive and active processes (Kochian, 1991). Nickel uptake is generally rapid and positively correlated with Ni concentrations in soil solution (Kabata-Pendias and Pendias, 1992). The presence of metal cations such as Zn^{2+}, Cu^{2+}, Ca^{2+}, or Mg^{2+} was observed to inhibit Ni^{2+} uptake in excised barley roots, but the effect was different between the macro and microelements, of competitive nature for Zn^{2+} and Cu^{2+} but non-competititve for Ca^{2+} and Mg^{2+} (Korner *et al*, 1986). Nickel uptake is influenced by many plant and soil factors as shown in Fig. 1 and discussed in the subsequent sections. pH is one of the most important soil properties, which decreases Ni uptake when raised from 4.5 to 6.5 (Kabata-Pendias and Pendias, 1992).

5.1.1. Bioactivation of nickel in the rhizosphere

A high proportion of metal-resistant bacterial populations live in the rhizosphere of the hyperaccumulators. The rhizosphere provides a complex and dynamic microenvironment where microorganisms, in association with roots, form a unique community (De-Souza *et al.*, 1999). Plant exudates, including organic acids (e.g., malonic and oxalic acids), metal-chelating compounds (phytosiderophores), and enzymes (reductase), together with acidification by protons, play a role in the metal mobilization from the soil to the roots (Marschner, 1995). In turn, rhizosphere microorganisms may interact symbiotically/non symbiotically with roots to enhance the potential for metal uptake through lowering the pH, producing compounds such as antibiotics and antifungals, organic acids, hormones (e.g., indole acetic acid), metal-chelating agents, enhancing plant biomass increases at root level (Wenzel *et al.*, 2003; Xiong *et al.*, 2008), solubilization of soil phosphate (Gupta *et al.*, 2002) and providing nutrients to the plant (Patten and Glick, 1996). Rhizosphere microbes, with activity and a high surface area-to-volume ratio because of their small size, provide a large contact area that may have the potential to act as microbial chelates associated with metal uptake (Karenlampi *et al.*, 2000; Rajkumar and Freitas, 2008a) (Table 1).

Extractable Ni was found to increase from 2.2 to 2.6 mg kg^{-1} when the soil was inoculated with *Microbacterium arabinogalactanolyticum* AY509224 (Abou-Shanab *et al.*, 2006). Plant growth-promoting bacteria (PGPB) *sychrobacter* sp. SRA1 and *Bacillus cereus* SRA10 significantly increased the accumulation of Ni in the root and shoot tissues of *Brassica juncea*, as compared with non-inoculated controls (Ma *et al.*, 2009a). These studies indicate that rhizosphere bacteria facilitate the release of Ni from the non-soluble phases in the soil, thus enhancing the availability of Ni to *A. murale* (Abou-Shanab *et al.*, 2003; 2006). Accordingly, Everhart *et al.* (2006) observed the highest number of Ni-resistant bacteria in the unlimed loam soil, which is also the soil that showed the highest Ni phytoextraction value. A possible explanation might be acid, siderophore production and phosphate solubilization by the bacteria, which facilitate Ni solubility (Abou-Shanab *et al.*, 2006).

In addition, some PGPB strains can produce siderophores and contain the enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, which can reduce the production of ethylene, thus protecting the plant against Ni, Pb, and Zn toxicity (Burd *et al.*, 1998), promoting root elongation and plant growth (Hall *et al.*, 1996; Glick *et al.*, 1998; Belimov *et al.*, 2001), and contributing to the heavy metal tolerance of plants. Burd *et al.* (2000) reported that Ni-resistant *Kluyvera ascorbata* isolated from soil contaminated with Ni and other heavy metals has been shown to promote plant growth. *Psychrobacter* sp. SRA2 significantly increased the fresh (351%) and dry biomass (285%) of the *B. juncea* (Ma

Table 1 Bacteria and plant combination in Ni phytoremediation of contaminated soils

Bacteria	Plants	Metals	Effects/Mechanisms	References
Kluyvera ascorbata SSUD165	Canola (*Brassica napus*)	Ni	Increased biomass; ACC deaminase	Burd *et al.*, 1998
K. ascorbata SUD165, SUD165/26	Canola, tomato (*Lycopersicon esculentum*)	Ni, Pb, Zn	Increased biomass; ACC deaminase, siderophores	Burd *et al.*, 2000
Microbacterium arabinogalactanolyticum	*Alyssum murale*	Ni	Increased Ni uptake	Abou-Shanab *et al.*, 2003
Rhizosphere and endophytic bacteria	*Thlaspi goesingense*	Ni	ACC deaminase, siderophores	Idris *et al.*, 2004
Rhizosphere bacteria	*Graminaceae grasses*	Cd, Zn, Ni	IAA, siderophores, ACC deaminase	Dell'Amico *et al.*, 2005
P. fluorescens, *P. putida*	Canola (*Brassica napus*)	Ni	Increased seed germination and growth	Ashour *et al.*, 2006
P. putida UW4, *P. putida* HS-2	Canola (*Brassica napus*)	Ni	Increased biomass in the field; IAA, ACC deaminase	Farwell *et al.*, 2006
B. subtilis SJ-101	Indian mustard (*Brassica juncea*)	Ni	Increased Ni uptake and Shoot length, fresh and dry weights; IAA, P solubilization	Zaidi *et al.*, 2006
Nine different Ni-resistant bacterial strains	*Alyssum murale*	Ni	Increased Ni uptake	Abou-Shanab *et al.*, 2006
P. putida ARB86	*Arabidopsis thaliana*	Ni	Increased biomass and chlorophyll content	Someya *et al.*, 2007

(*continued*)

Table 1 (*continued*)

Bacteria	Plants	Metals	Effects/Mechanisms	References
Bradyrhizobium sp. RM8	Mung bean (*Vigna mungo*)	Ni, Zn	Increased growth, seed yield and seed protein, nodule number and plant Nutrition, decreased Ni toxicity and uptake; IAA, siderophores, HCN, ammonia production	Wani *et al.*, 2007a
Methylobacterium oryzae CBMB20, *Burkholderia sp.*	*Lycopersicon esculentum*	Ni, Cd	Increased Plant growth, decreased Ethylene emission, and Ni, Cd Uptake and translocation; ACC deaminase, phytohormone production	Madhaiyan *et al.*, 2007
Endophytic bacteria	*A. bertolonii*	Ni	Increased biomass; siderophores	Barzanti *et al.* (2007)
Rhizobium sp. RP5	*Pisum sativum*	Ni, Zn	Increased biomass, nodule number and plant nutrition, decreased Ni, Zn uptake and toxicity; IAA, siderophores	Wani *et al.*, 2007b
Enterobacter sp. NBRI K28	Indian mustard (*Brassica juncea*)	Ni, Zn, Cr	Increased biomass, protein and chlorophyll content and Ni, Zn, Cr uptake; IAA, siderophores, ACC deaminase, P solubilization	Kumar *et al.*, 2008
P. putida HS-2	Canola (*Brassica napus*)	Ni	Increased seed germination and biomass; siderophores, IAA, ACC deaminase	Rodriguez *et al.*, 2008

Pseudomonas sp. 29C, *Bacillus sp.* 4C	Indian mustard (*Brassica juncea*)	Ni	Increased biomass; IAA, iderophores, ACC deaminase, P solubilization	Rajkumar And Freitas, 2008a
Pseudomonas sp. M6, *Pseudomonas jessenii* M15	Castor bean (*Ricinus communis*)	Ni, Cu, Zn	Increased biomass; IAA, ACC deaminase, P solubilization	Rajkumar And Freitas, 2008b
Pseudomonas sp.	Chickpea (*Cicer arietinum*)	Ni	Increased biomass and decreased Ni uptake; siderophores	Tank and Saraf, 2008
Enterobacter erogenes, *Rahnella aquatilis*	Indian mustard (*Brassica juncea)*	Ni, Cr	Increased biomass, and Ni, Cr uptake; IAA, siderophores, ACC deaminase, P solubilization	Kumar *et al.*, 2009
Psychrobacter sp. SRA1 and SRA2, *Bacillus cereus* SRA10	Indian mustard (*Brassica juncea*), *B. oxyrrhina*	Ni	Increased biomass, and Ni bioavailability and uptake; IAA, siderophores, ACC deaminase, P solubilization	Ma *et al.*, 2009a
Pseudomonas sp. SRI2, *Psychrobacter sp.* SRS8 and *Bacillus sp. SN9*	Indian mustard (*Brassica juncea*), *B. oxyrrhina*	Ni	Increased biomass, and Ni bioavailability and uptake; IAA, siderophores, ACC deaminase, P solubilization	Ma *et al.*, 2009b
endophyte *Burkholderia cepacia* VM1468	yellow lupine	Ni	Decreased Ni and toluene phytotoxicity	Weyens *et al.*, 2010

(Sources: Glick, 2010 and Ma *et al.*, 2011)

et al., 2009a). Besides, Rajkumar and Freitas (2008a) reported inoculation with the Ni-resistant PGPB, *Pseudomonas sp.* 29C and *Bacillus megaterium* 4C, had little influence on the accumulation of Ni in root and shoot system, but produced a much larger above-ground biomass of *B. juncea*. This observation showed that the strains Ps29C and Bm4C protect the plants against the inhibitory effects of Ni, probably due to the production of indole-3-acetic acid (IAA), siderophore and solubilization of phosphate. Some other bacteria belonging to different genera such as pseudomonas, mycobacterium, agrobacterium, and arthrobacter are also reported to produce IAA and aid in plant growth (Dell'Amico *et al.*, 2005).

Amino cyclopropane-1-carboxylic acid deaminase (AcdS) is an enzyme that is involved in the hydrolysis of plant hormone ethylene precursor ACC. The AcdS activity of many rhizobacterial strains may decrease the level of ethylene in root tissue, thereby promoting root growth (Glick *et al.*, 1998; Contesto *et al.*, 2008). Contesto *et al.* (2008) investigated the changes in root architecture and root hair length induced by four rhizobacteria (*Phyllobacterium brassicacearum* STM196, *Pseudomonas putida* UW4, *R. leguminosarum* bv. *viciae* 128C53K and *Mesorhizobium loti* MAFF303099) and by their respective AcdS-deficient mutants. The results showed that inoculation by any of the rhizobacteria led to a 2—3-fold increase in root hair length in *A. thaliana*. By contrast, root hairs of seedlings inoculated with the AcdS mutant strains were significantly increased. Similar effects have been reported for many other PGPR strains (Zimmer and Bothe, 1988; Bertrand *et al.*, 2000; Dobbelaere *et al.*, 2003; Ribaudo *et al.*, 2006).

Maestri *et al.* (2010) stated that root environment for hyperaccumulator and non-accumulator plant species seems to have different effects on metal bioavailability and root uptake, but more studies are needed to ascertain this.

5.1.2. Root absorption and compartmentation

In root the plasma membrane (PM) is the first structure of living cells exposed to heavy metal. The PM functions as a barrier for metal movement into the cytoplasm (Kabała *et al.*, 2008). A key PM enzyme, H^+-ATPase, generates the proton electrochemical gradient, which acts as a driving force for nutrient uptake and loading of solutes and assimilates into the xylem and phloem, respectively (Morsomme and Boutry, 2000; Palmgren, 2001; Kabała *et al.*, 2008). Studies have shown that the increase or decrease of the PM H^+-ATPase activity in roots depends greatly on the metal concentration in the cultivation medium (Ros *et al.*, 1992b; Burzyński and Buczek, 1994; Janicka-Russak *et al.*, 2008).

In order to enter the xylem, Ni must first cross a membrane, probably through the action of membrane pump or channel. This type of transport of metals, called symplast transport, is regulated due to the selectively

permeable plasma membrane of the cells that control access to the symplast by specific or generic metal ion carriers or channels (Gaymard, 1998; Hall, 2002; Sheoran *et al.*, 2009). Soluble Ni can enter into the root symplast by crossing the PM of the root endodermal cells or they can enter the root apoplast through the space between cells (Peer *et al.*, 2005; Mukhopadhyay and Maiti, 2010). Few data are available on the kinetic parameters of Ni uptake by vascular plant roots (Cataldo *et al.*, 1978; Verlière and Heller, 1981; Aschmann and Zasoski, 1987; Puschenreiter *et al.*, 2005). Most research on trace element uptake kinetics has focused on symplastic influx parameters, considering the apoplastic uptake to play no role in trace-element uptake. This is because apoplastic trace elements are considered to be blocked in the roots behind the apoplastic barriers (Casparian band), and therefore not translocated into the shoots (Redjala *et al.*, 2010). However, a recent study using the fractionation method, allowed a separate assessment of the kinetics of apoplastic and symplastic Ni uptake for maize and *Leptoplax emarginata* with contrasting demands for the metal, revealed the importance of Ni adsorption on root apoplast (representing 81–95% of the total root uptake of both plant species), and suggested that apoplastic adsorption might also compete with the symplastic absorption and adds to the amount of Ni taken up by the plant, particularly in roots (Redjala *et al.*, 2010).

5.2. Root-to-Shoot Transport

Plant uptake of Ni is accomplished by passive diffusion and active transport but their relative contribution depends on Ni concentration (Seregin and Kozhevnikowa, 2006). The active transport is more important at low Ni concentrations (below 34 $\mu mol\,L^{-1}$), and the diffusion process increases its role at higher concentrations due to the Ni toxic effect (Demchenko *et al.*, 2005; Jean *et al.*, 2008).

Unlike non-hyperaccumulator plants, which retain in root cells most of the heavy metals taken up from the soil, detoxifying them by chelation in the cytoplasm or storing them into vacuoles, in hyperaccumulators, once loaded into the xylem, the flow of xylem sap can rapidly and efficiently transport Ni to the shoots (Maestri *et al.*, 2010). Metal transporters carry metal ions from root symplast into xylem apoplast (Marschner, 1995), and are probably driven by transpiration pump (Salt *et al.*, 1995b). Although detailed information on the molecular factors governing metal translocation is still lacking, a number of different plant metal transporter families, as well as natural chelators and organic acids, have been identified to be effective in enhancing metal uptake and translocation from roots to the shoots in hyperaccumulators.

Lasat *et al.* (2000) and Rascio and Navari-Izzo (2011) concluded that the xylem translocation entails the heavy metal availability for xylem loading,

which derives from a low sequestration into and a ready efflux out of the vacuoles, possibly due to the specific features of root cell tonoplast. Xylem loading is known to operate through cation-proton antiport, cation-ATPases or ion channel (Williams *et al.*, 2000; Yang *et al.*, 2005).

5.2.1. Organic acids

Whether the long distance xylem transport of heavy metals can occur in free ionic forms or through metal complexation with organic acids is still controversial (Rascio and Navari-Izzo, 2011). It has been reported that only one-third of Ni has been bound by citrate in the xylem of hyperaccumulator *Stackhousia tryoni* (Celastraceae), whereas in the latex of the extreme Ni hyperaccumulator *S. acuminata* plant almost all Ni is complexed with citrate and other organic acids (Bidwell, 2001; Callahan *et al.*, 2008; Rascio and Navari-Izzo, 2011). In several hyperaccumulating plant species, Citric acid was found to be the predominant chelator for Ni transport (Lee *et al.*, 1977, 1978; Kersten *et al.*, 1980). Studies with *Alyssum bertolonii* showed that Ni uptake was enhanced *via* Ni complexation with citric acid (Boominathan and Doran, 2003b). Moreover, in *Datura innoxia,* Ni is mainly present in the roots, nevertheless, Ni concentrations in stems of *D. innoxia* increased with the higher citric acid concentrations (Jean *et al.*, 2008).

A key role of organic acids in heavy metal hyperaccumulation seems to be related to free amino acids, such as His and nicotinamine (NA), which form stable complexes with bivalent cations and free His appears to be the most important ligand involved in Ni hyperaccumulation (Callahan *et al.*, 2006; Rascio and Navari-Izzo, 2011). In the Ni hyperaccumulator *Alyssum lesbiacum*, free His has been identified as the only amino acid shown to increase tolerance and translocation of metals to the shoot (Krämer *et al.*, 1996). In *Alyssum montanum,* Ni is transported into the xylem at a 50-fold increased rate by chelating with His (Krämer *et al.* 1996). The concentration of His in *A. lesbiacum* is increased by Ni treatment, but the genes of the biosynthetic pathway are not induced (Persans *et al.*, 1999; Ingle *et al.*, 2005). In the roots of Ni hyperaccumulator *A. lesbiacum*, as compared with the non-hyperaccumulator *A. montanum*, the constitutive overexpression of the TP-PRT1 gene (encoding the ATP-phosphoribosyl transfer enzyme, committed in the first step of the biosynthetic pathway) leads to a larger endogenous pool of His, which favors the Ni xylem loading as a Ni—His complex (Kerkeb and Krämer, 2003; Ingle *et al.*, 2005). Results with other species of the *Brassicaceae* family suggest that His-dependent Ni xylem loading may not be universal (Persans *et al.*, 1999; Montargès-Pelletier *et al.*, 2008; Verbruggen *et al.*, 2009). Araújo *et al.* (2009) investigated the influence of four different ligands present in the xylem sap of *Quercus ilex* (His, citric, oxalic and aspartic acids) on Ni adsorption by xylem. The data fit

the Freundlich isotherm model, the decreasing affinity order of ligands for Ni was: oxalic acid > citric acid > His > aspartic acid. On the other hand, the Ni adsorption by xylem increased following the inverse sequence of ligands (Araújo *et al.*, 2009). Besides, Wycisk *et al.* (2004) demonstrated that enhancing the first step in the His biosynthesis pathway, by expression in *Arabidopsis thaliana* of a bacterial gene encoding ATP phosphoribosyl transferase, is sufficient to increase the endogenous free His pool and Ni tolerance in *A. thaliana*. Hyperaccumulators contain a larger pool of His available for chelation in roots. The formation of Ni—His complex in the cytoplasm of root cells plays an essential role in preventing Ni entrapment in root vacuole, thus keeping it in the cytosol, in a detoxified form to facilitate its translocation (Maestri *et al.*, 2010). This could be the probable reason for higher rate of Ni loading in the xylem sap of *Thlaspi caerulescens*, as compared with the non-accumulator *Thlaspi arvense* (Richau *et al.*, 2009; Maestri *et al.*, 2010).

Nicotinamine is synthesized by the enzyme NA synthase from three molecules of S-adenosylmethionine, whereby three molecules of 50-S-methyl-50-thioadenosine are released (Wycisk *et al.*, 2004). Nicotinamine forms complexes with most transition metal ions and its role is supposed to facilitate the movement of micronutrients throughout the plant (Stephan and Scholz, 1993). Studies suggested that NA is involved in iron homeostasis and confers Zn (Becher *et al.*, 2004; Weber *et al.*, 2004) and Ni tolerance (Vacchina *et al.*, 2003). Positive correlation exists between enhanced NA synthesis and NA-metal chelation with Ni hyperaccumulation in *T. caerulescens* (Vacchina *et al.*, 2003). After 6 h of exposure to Ni, TcNAS1 was expressed in shoots, NA was found in roots, and NA—Ni complexes became detectable in the xylem sap (Maestri *et al.*, 2010). NA seems to be translocated to the roots in response to Ni toxicity, where it chelates with the absorbed Ni, thus facilitating NA—Ni complex transport to the shoot (Maestri *et al.*, 2010). Through liquid chromatograpy coupled with electrospray ionization mass spectrometry, NA was found to play a crucial role in Ni translocation within metal-accumulating plants (Mari *et al.*, 2006; Ouerdane *et al.*, 2006; Callahan *et al.*, 2007).

Other known metal chelators, such as phytochelatins (PCs) or glutathione (GSH), are not involved in Ni binding in the Ni hyperaccumulators such as *A. lesbiacum* (Krämer *et al.*, 1996) and *Thlaspi goesingense* Hálácsy (Krämer *et al.*, 2000).

5.2.2. Transport proteins

Each transporting protein usually catalyzes the transport of several ions, but with different affinity. Specific transporters in plant responsible for Ni hyperaccumulation have not yet been recognized (Rascio and Navari-Izzo, 2011). Assunção *et al.* (2008) stated that the preference of Zn over Ni

absorption by some Zn- and Ni-hyperaccumulator plants supplied with the same concentration of both heavy metals strongly suggests that a Zn transport system might also be employed in Ni entrance into roots.

In metal transport, ZIP and NRAMP play an important role (Guerinot, 2000; Williams *et al.*, 2000). Mizuno *et al.* (2005) have reported that ZIP and RAMP transporter genes (*TjZnt1*, *TjZnt2* and TjNRAMP4) from the sole Ni hyperaccumulator (*Thlaspi japonicum*) found in Japan were cloned and their Ni-transport ability was demonstrated by expression in yeast. Further, the ZIP and NRAMP transporters, isolated from *T. japonicum,* appear to protect cells from high-Ni levels. Transporter genes, TjZNT1 and TjZNT2 contain two long His-rich domains (HRDs) and act as a strong Ni-binding region. Mizuno *et al.* (2007) further reported that introduced ZIP family transporter gene homologous to *TjZnt1* and *TjZnt2* into yeast strains increased Ni tolerance in that species. However, the action of ZIP family transporter homologous could not explain the Ni resistance of yeast strains transformed with *TjZnt1* and *TjZnt2*. Mizuno *et al.* (2007) found that Ni tolerance of the *TjZnt2* expressing yeast strain was not correlated with binding to HRDs, and Ni tolerance of *TjZnt1*; however, it was partially correlated with Zn influx, which suppressed Ni influx, therefore Ni influx and competitive inhibition of Ni influx by other metals might be the possible explantation. NRAMP family acts to control and maintain homeostasis of metals, with location mainly at the vacuolar membrane (Mizuno *et al.*, 2005). In contrast to the wide metal transport specificity of TjZNT1 and TjZNT2 that transport Ni, Mn, Zn, and Cd, TjNRAMP4 did not show any transport activity for metals other than Ni (Mizuno *et al.*, 2005).

In *T. caerulescens*, TcYSL3 was identified to participate in the vascular loading and mediate translocation of the Ni—NA complex *via* symplast (Gendre *et al.*, 2007). HMAs is of particular importance in heavy metal transport and plays a role in metal homeostasis and tolerance (Axelsen and Palmgren, 1998). Interestingly, the *HMA4* (one of the genes coding bicalent cation transporters of HMAs) activity has been shown to positively affect other candidate genes for hyperaccumulation. The increased expression of HMA4 increased the expression of genes belonging to the *ZIP* family, further suggesting that the root-to-shoot translocation acts as a driving force of the hyperaccumulation, by creating a permanent metal deficiency response in roots (Hanikenne *et al.*, 2008; Rascio and Navari-Izzo, 2011).

5.3. Distribution/Detoxification/Sequestration

5.3.1. Distribution

The knowledge of spatial distribution and localization of Ni and other metals within plant tissues is needed to comprehend mechanisms and

ecological benefits of hyperaccumulation and tolerance to toxic levels of heavy metals. For instance, only when Ni is present at sufficiently high concentrations in the phloem, can protection against phloem-sucking insects be effective (Gramlicha *et al.*, 2011).

It has been extensively reported that the higher concentration of Ni was found in the above-ground parts of plants rather than in the roots (Shallari *et al.*, 1998; Broadhurst *et al.*, 2004; Bani *et al.*, 2007). For instance, *A. murale* grown in ultramafic soil near Pogradec in Albania, seeds contained the highest Ni concentration (stems: 1170 mg kg^{-1}, roots: 1260 mg kg^{-1}, and seeds: 11,400 mg kg^{-1}) (Barbaroux *et al.*, 2009). Also, *A. murale* has been shown to contain a high amount of Ni in the leaves at the flowering stage (Bani *et al.*, 2007; Chaney *et al.*, 2007). Mesjasz-Przybylowicz *et al.* (1994) reported that for *S. coronatus,* most of the Ni was located in leaves and was concentrated mostly in the leaf epidermis. Ni-hyperaccumulating plants are known to contain Ni concentrations from several thousands of mg kg^{-1} up to 5% Ni in leaf dry matter (Baker *et al.*, 1992; Prasad, 2005). Sander and Ericsson (1998) found that concentrations of Zn, Cu, Ni, and Cd in stems of *Salix viminalis* increased significantly with plant height, thought principally to be a consequence of increasing bark proportions.

A significantly higher Ni concentration in the leaves than in the stems and roots (leaves > stems > roots) has been reported in the Ni hyperaccumulator *Berkheya coddii* by using different methods (Mesjasz-Przybylowicz *et al.*, 2001; Robinson *et al.*, 2003; Budka *et al.*, 2005; Moradi *et al.*, 2010; Gramlicha *et al.*, 2011), the highest concentrations were in the lower leaves and upper stems. In the stems of *B. coddii*, Ni concentrations were higher in the epidermis than in the cortex and the pith, and the vascular bundles occasionally showed higher Ni accumulation than cortex and pith (Budka *et al.*, 2005; Gramlicha *et al.*, 2011). Nickel concentrations in leaves of *B. coddii*, were lower in the inner collenchymas and the vascular bundles than in the upper epidermis. In the top leaves, the lower epidermis showed significantly lower concentrations than the upper epidermis (Gramlicha *et al.*, 2011). The high concentrations were found in the midrib of *B. coddii* (Mesjasz-Przybylowicz *et al.*, 2001; Gramlicha *et al.*, 2011). In the roots of *B. coddii*, the lowest root Ni concentrations were observed in the stele (Moradi *et al.*, 2010; Gramlicha *et al.*, 2011). Nickel distribution patterns similar to this have been reported in the roots of other Ni hyperaccumulators (Gramlicha *et al.*, 2011). In roots of Ni-hyperaccumulator plant *S. coronatus* Harv the concentrations of Ni decreased from the outer cortex toward the inner cortex (Mesjasz-Przybylowicz *et al.*, 2007), and in *Stackhousia tryonii* F.M. Bailey plants higher Ni-concentrations were found in the vascular bundles than in the cortex and pith (Bhatia *et al.*, 2004). High Ni-concentrations in epidermal tissues were observed in *A. bertolonii* Desv plants (Marmiroli

et al., 2004). Nickel was found to be located within the epidermal cells or in the basal compartment or pedicle of the trichomes on the leaf surface in *A. lesbiacum, A. bertolonii, A. murale, L. emarginata*, and *T. gosingense* (Krämer *et al.*, 1997; Psaras *et al.*, 2000; Broadhurst *et al.*, 2004). Additionally, Hasselgren (1999) determined that Cu, Pb, and Cr were mainly found in the stems, while Zn, Cd, and Ni were found mainly in the leaves of sludge-amended willow plots.

Nevertheless, different Ni distribution patterns were observed in other plant species. For example, Marques *et al.* (2009) reported that in *Rubus ulmifolius,* Ni was only distributed in the root. Berazain *et al.* (2007) found the highest Ni-concentrations in the cortex of the stems of six species of the Euphorbiaceae family. In an endemic serpentinic Proteaceae of New Caledonia *Grevillea exul* var. exul, Ni was highest in the roots, lower in stems with a higher accumulation in the basal part than in the upper one and, not detectable in leaves. Nickel was concentrated mostly in the phloem compared to the xylem and the epidermis, either in roots or stems and was mostly in the epidermis in the upper part of the stems (Rabier *et al.*, 2007; 2008). *S. acuminata* (Sagner *et al.*, 1998) and *Q. ilex* (Nabais *et al.*, 1996) tree species accumulated Ni in the conducting tissues, while Ni is mainly localized in the leaf epidermis of herbaceous species (Broadhurst *et al.*, 2004; Heath *et al.*, 1997) or shrubs (Severne, 1974). Rabier *et al.* (2008) concluded that the difference of Ni localization in roots, stem, and leaves tended to reflect the existence of different tolerance strategies among herbaceous bushes and trees species for storage in senescing tissues rather than the simple distinction between excluders and accumulators.

Although extensive work has been performed on Ni-hyperaccumulator plants in trying to determine the principal sites of metal localization within plant tissues, no consistent picture of Ni distribution emerged. This might be caused by differences of plant species, as well as conditions of cultivation, and the developmental age of analyzed tissues (Grovenor *et al.*, 2006; Smart *et al.*, 2007; Bhattacharyya *et al.*, 2008). In *B. coddii*, Ni is redistributed from older to younger leaves through phloem transport (Marschner, 1995; Page and Feller, 2005; Riesen and Feller, 2005). Moreover, in recent years, many methods have been used to analyze Ni distribution in hyperaccumulator plants, such as electron dispersive X-ray analysis (EDXA) (Robinson *et al.*, 2003; Bidwell *et al.*, 2004; Marmiroli *et al.*, 2004; Berazain *et al.*, 2007), quantitative micro-proton induced X-ray emission spectroscopy (μ-PIXE) (Mesjasz-Przybylowicz *et al.*, 2001; Bhatia *et al.*, 2004; Budka *et al.*, 2005; Kachenko *et al.*, 2008), high-resolution secondary ion mass spectroscopy (NanoSIMS) (Smart *et al.*, 2007), synchrotron-based fluorescence computed microtomography (CMT) (McNear *et al.*, 2005), dimethylglyoxime (DMG) (Mizuno *et al.*, 2003; Seregin *et al.*, 2003; Bhatia *et al.*, 2004; Richau *et al.*, 2009; Gramlicha *et al.*, 2011), and inductively

coupled plasma-mass spectroscopy (ICP-MS) (Marques *et al.*, 2009). However, each method has its unique advantages and disadvantages such as the detection limit and the redistribution of Ni during sample pretreatment. For example, EDXA may respond differently to less soluble Ni fractions than DMG (Gramlicha *et al.*, 2011). Establishment of a uniform detection system with sensitive detection limits and preparation methods that do not modify the sample will be necessary for investigation of the spatial distribution and localization of Ni within plant tissues.

5.3.2. Sequestration and detoxification

Great efficiency in detoxification and sequestration is a key property of hyperaccumulators which allows them to concentrate huge amounts of heavy metals in above-ground organs without suffering any phytotoxic effect (Rascio and Navari-Izzo, 2011). A general mechanism for detoxification of heavy metals in plants is the distribution of metals to cellular locations where the metal will not damage the vital cellular processes such as photosynthesis, and chelation of the metals by ligands, followed by the sequestration of the metal–ligand complex from metabolically active cytoplasm into inactive subcellular compartments such as vacuoles and cell walls. The sequestered metal in shoot cells can be in the apoplast or in specialized cell types, such as epidermal cells (Heath *et al.*, 1997; Bidwell *et al.*, 2004; Ma *et al.*, 2005; Asemaneh *et al.*, 2006; Freeman, *et al.*, 2006), mesophyll cells (Hale *et al.*, 2001), trichomes (Küpper *et al.*, 2000; 2001), and cuticle (Robinson *et al.*, 2003). It was reported that, localization in epidermal cells is an important physiological mechanism involved in Ni accumulation and tolerance, in leaves of Ni hyperaccumulating shrub-*Hybanthus floribundus* (Kachenko *et al.*, 2008). Nickel ions taken up by roots of *H. floribundus* are complexed by ligands (Kersten *et al.*, 1980). Following complexation, Ni is compartmentalized within epidermal tissues as the preferential site of Ni accumulation with high concentrations in epidermal vacuoles (Bidwell *et al.*, 2004).

Nickel accumulates primarily in the cell vacuoles of epidermal stem and leaf tissues and trichome basal compartments (Broadhurst *et al.*, 2004; McNear *et al.*, 2005; McNear *et al.*, 2010). At higher growth medium Ni concentrations, accumulation by palisade mesophyll cells becomes important probably due to overflow of the primary cellular storage compartments (Broadhurst *et al.*, 2004; McNear *et al.*, 2010). In many cases heavy metals are also excluded from both subsidiary and guard cells of stomata (Frey *et al.*, 2000; Broadhurst *et al.*, 2004; Cosio *et al.*, 2005). This may preserve the functional stomatal cells from metal phytotoxic effects (Rascio and Navari-Izzo, 2011). Cell walls may play an important role in detoxifying metals in plant cells of the Ni and Zn/Cd hyperaccumulating plant

species. About 60–70% of accumulated Ni and/or Zn are distributed in the apoplast cell walls (Krämer *et al.*, 2000; Yang *et al.*, 2005; Li *et al.*, 2006). However, molecular basis of metal detoxification by cell walls is not well understood (Yang *et al.*, 2005). The precise localization of hyperaccumulated metals and their possible binding with cell walls are still debated as the situation is complicated because different analytical techniques involve different preparation operations that may affect the status of metals (Montargès-Pelletier *et al.*, 2008).

Invariably, in most of the Ni-hyperaccumulator plants, the typical Ni concentration distribution follow the order of Ni in shoots (leaves > stems) > roots; however storage in senescing root tissues might be a supplementary mechanism of Ni detoxification besides metal accumulations in shoots (Rabier *et al.*, 2008). Even in the hyperaccumulator species *Psychotria douarrei* known for its high-Ni accumulation in leaf, a very high level of Ni (92,500 ppm) were observed in the bark of the root (Jaffré and Schmid, 1974; Rabier *et al.*, 2008). The high amount of Ni in the roots and the poor translocation to the leaves in *D. innoxia* may be explained by a sequestration of Ni on the cation exchange sites of the xylem parenchyma vessel walls in roots and immobilization in the vacuoles of the root cells (Jean *et al.*, 2008). Plant ability to tolerate heavy metals is probably due to the capacity of cell walls to bind metals (Seregin and Kozhevnikowa, 2006). Jean *et al.* (2008) stated that Ni could be fixed in the stems of *D. innoxia* because of the existence of equilibrium between metal, chelates and the metal binding sites in the cell wall of the xylem vessels.

5.3.2.1. Vacuolar sequestration

Vacuolar sequestration is considered to play an important role in plant metal homeostasis and plant detoxification from heavy metals (Martinoia *et al.*, 2007). The vacuole is generally considered the main storage site for metals in yeast and plant cells, and it has been shown that PC-metal complexes are pumped into the vacuole in plants (Salt *et al.*, 1995a,b) and in fission yeast (*Schizosaccharomyces pombe*) (Ortiz *et al.*, 1995) and in plants (Salt *et al.*, 1995a,b). Moreover, it is considered the vacuolar Ni storage in leaf cells is the main biochemical detoxification mechanism by the comparing Ni compartmentation between non-accumulating and hyperaccumulating species (Krämer *et al.*, 2000). Compartmentalization of metals in vacuoles is very effective in controlling the distribution and concentration of metal ions. Wu *et al.* (2010) stated that vacuole compartmentalization "arrest and imprisons" hazardous metal ions, constricting them into a limited site where other parts of the cell have no access to those toxic metal ions and safety is ensured.

Metal ions sequestration inside the vacuole involves active transport systems operating in the tonoplast. Transport of heavy metals across the

tonoplast is energized directly by ATP hydrolysis such as primary active transporters that includes ABC-type proteins and P1B-ATPases or by the transmembrane pH gradient such as secondary active antiporters (Krämer *et al.*, 2007; Martinoia *et al.*, 2007). Tonoplast antiporters are responsible for compartmentalization of metals in the vacuoles and these have been identified and classified to the CDF, CAX and magnesium exchangers (Shaul *et al.*, 1999; Kobae *et al.*, 2004; Shigaki and Hirschi, 2006). The central vacuole is considered as the site of Ni sequestration. Some tonoplast efflux transporters are required to transport excess Ni present in the cytosol into the vacuole against their electrochemical gradient (Kabała and Janicka-Russak, 2011). Krämer *et al.* (2000) observed that by "imprisoning" most of the intracellular leaf Ni into vacuole, Ni tolerance of hyperaccumulator *T. goesingense* is greatly improved. In this plant, high expression of MTP1 tonoplast protein has been reported to confer tolerance to Ni as well as to Cd and Co (Persans *et al.*, 2001).

The presence of Ni^{2+}/H^{+} antiport system has been demonstrated in the tonoplast of hyperaccumulator species-*A. lesbiacum* (Ingle *et al.*, 2008). Moreover, it was reported that in the yeast *Saccharomyces cerevisiae* vacuolar accumulation of Ni is essential for Ni resistance (Ramsay and Gadd, 1997; Nishimura *et al.*, 1998; Yang *et al.*, 2005). Such vacuolar accumulation of Ni in yeast is driven by the pH gradient that exists across the vacuolar membrane (Nishimura *et al.*, 1998; Yang *et al.*, 2005). In roots of Ni sensitive oat seedlings, surprisingly, Ni-transport dependence upon the pH gradient was not confirmed (Gries and Wagner, 1998); however, only a minor accumulation of Ni could be detected in vacuoles isolated from leaves of Ni sensitive barley.

An electrochemical gradient across the tonoplast, required for metal accumulation, is generated by two proton pumps, vacuolar-proton transporting ATPase (V-ATPase) and Vacuolar H^{+}-translocating pyrophosphatase (V-PPase) (Kabała and Janicka-Russak, 2011). These proton pumps seem to be an important element of the mechanisms involved in plant metal tolerance. V-ATPase and V-PPase generate an electrochemical proton gradient across the tonoplast, energize secondary transporters operating as H^{+}-coupled carriers, including metal efflux transporters (Kabała and Janicka-Russak, 2011). Lower concentration of Ni stimulated V-PPase activities, but did not significantly change the action of V-ATPase, while, higher-metal concentration had visible inhibitory effect on the functioning of V-ATPase, especially on the ATP-driven proton transport across the tonoplast (Kabała and Janicka-Russak, 2011). In addition, treatment of plants with Ni stimulated the activity of PM H^{+}-ATPase in shoots (Ros *et al.*, 1990; 1992a; 1992b). Therefore, it was concluded that the effect of Ni on ATP and PPi-driven proton pumping in tonoplast vesicles depended on the metal concentration in the medium and correlated well with metal accumulation in the root tissues

(Kabała and Janicka-Russak, 2011). An inhibitory effect on V-ATPase and V-PPase activities was observed when Ni was applied *in vitro* at high levels to *Cucumis sativus* roots (Kabała and Janicka-Russak, 2011). Comparable observations were made by Kennedy and Gonsalves (1989) and Ros *et al.* (1992b) for the plasma membrane ATPase with Ni. These results suggest that different mechanisms may be responsible for heavy metal action *in vivo* and *in vitro* roots (Kabała and Janicka-Russak, 2011).

5.3.2.2. Chelators

Wu *et al.* (2010) stated that heavy metal chelation (binding of metal ions by ligands) plays a crucial role not only in the accumulation and transportation of heavy metals, but also in the detoxification phase. Chelated metal ions are uncharged and inert to react with other substances, therefore cell damage by heavy metals is reduced significantly. Once metals are taken up into the cytosol, and in particular in vacuoles, metal ions can be chelated by different ligands such as organic acids (oxygen donor ligands), amino acids (oxygen and nitrogen donor ligands), metallothioneins (MTs) (sulfur donor ligands), PCs (peptides, oxygen and nitrogen donor ligands), and other high molecular weight molecules (proteins, chaperones).

Small ligands, such as organic acids, may be instrumental in prevention and persistence of heavy metals as free ions in cytoplasm. These ligands enable entrapment of heavy metals in vacuoles where the metal-organic acid chelates are primarily located (Rascio and Navari-Izzo, 2011). In leaves of *T. goesingense,* for instance, citrate is the main ligand for entrapment of Ni (Krämer *et al.*, 2000). Moreover, in *H. floribundus*, the majority of Ni (up to 95%) was bound to citrate in leaf tissue (Kersten *et al.*, 1980). A correlation between Ni and citric acid concentrations in leaves has been demonstrated in a range of hyperaccumulating species (Lee *et al.*, 1978). In the case of Ni hyperaccumulators-*Phyllanthus serpentinus* and *P. douarrei*, Ni-malate was shown to be the dominating chemical form of Ni within the plant cells (Kersten *et al.*, 1980). It has been reported that in *S. acuminata* latex between 37% and 99% of the Ni is complexed by citric acid (Sagner *et al.*, 1998; Schaumlöffel *et al.*, 2003). Schaumlöffel *et al.* (2003) stated that as citrate does not bind Ni strongly ($\lg K = 5.4$, where K is the association constant of the complex), the Ni ion may be transported to the laticifers in the form of its more stable complexes with NA ($\lg K = 16.1$). Recently, a new Ni ligand, methylated aldaric acid (2, 4, 5-trihydroxy-3-methoxy-1, 6-hexan-dioic acid), was identified for the first time in biological extracts. After citric acid, methylated aldaric acid appears to be one of the most abundant small organic molecules present in the latex of the hyperaccumulating tree *S. acuminata* (Callahan *et al.*, 2008). The transport and storage of Ni in several Alyssum species has been attributed to organic acids and

possibly His (McNear Jr. *et al.*, 2010). Montargès-Pelletier *et al.* (2008) found that *L. emarginata*, *A. murale*, and *T. caerulescens* stored Ni in their leaves as Ni-malate and in the stems as Ni-citrate complexes, while His could not be detected either in leaves, stems nor roots of these three plants. McNear Jr. *et al.* (2010) suggested that Ni is concentrated in the dermal leaf and stem tissues of *A. murale* bound primarily to malate along with other low-molecular-weight organic acids (LMWOA) and possibly counter anions (e.g., sulfate). Nickel is present in the plant sap and vasculature bound to His, malate and other low molecular weight compounds. The data presented in the article by McNear *et al.* (2010) supports a model in which Ni is transported from the roots to the shoots complexed with His and stored within the plant leaf dermal tissues complexed with malate, and other low molecular weight organic acids or counter-ions.

In some plants, the Cys-rich peptide ligands MTs and PCs have been shown to play a role in metal tolerance when accumulated metals are at phytotoxic levels, but less so in metal tolerant plants (Schat *et al.*, 2002). Ag (I), Cd (II), Co (II), Cu (II), Hg (II), and Ni (II) metals are sequestered by binding with organic sulfur (R-SH) on the Cys residues of these peptides (Eapen and D'Souza, 2005). Family of MTs genes in plants encoding peptides that are generally composed of 60–80 amino acids and contain 9–16 Cys residues and these MT genes can protect plants from toxic metal ions (Zhou and Goldsbrough, 1995; Chatthai *et al.*, 1997). These MT-metal complexes can be glutathionated, suggesting that they may be transported into vacuoles for long-term sequestration (Brouwer *et al.*, 1993; Eapen and D'Souza, 2005). PCs, composed of only three amino acids, glutamine (Glu), Cys and glycine (Gly), are used in algae and plants to detoxify excess cellular metal (Zenk, 1996). PCs complex with metals and facilitate their storage in vacuoles. PCs are rapidly induced in cells and tissues exposed to a range of heavy metal ions, such as Cd, Ni, Cu, Zn, Ag, Hg, and Pb, and anions, such as arsenate and selenite (Rauser, 1995; Yang *et al.*, 2001). Nevertheless, their production in hyper-tolerant species is only inducible at extremely high exogenous metal concentration (Ebbs *et al.*, 2002; Schat *et al.*, 2002). Raab *et al.* (2004) and Schat *et al.* (2002) suspected that the heavy metal detoxification in hyperaccumulators, in contrast with tolerant non-hyperaccumulator plants, does not rely on these high molecular mass ligands. Antioxidation-related genes overexpression (Chiang *et al.*, 2006), and enhanced synthesis of GSH are pivotal antioxidant molecules (van de Mortel *et al.*, 2008), involved in a plethora of cellular processes in hyperaccumulators, including defense against ROS rise due to heavy metal stress (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), and sequestration and detoxification of heavy metals (Cobbett and Goldsbrough, 2002; Freeman *et al.*, 2004). GSH has been

detected virtually in all cell compartments such as cytosol, chloroplast, endoplasmic reticulum, vacuole, and mitochondria (Yadav, 2010). GSH represents one of the major sources of non-protein thiols in most plant cells. In plants, during degradation of H_2O_2, change in the ratio of its reduced (GSH) to oxidized (GSSG) form is important in certain redox signaling pathways (Millar *et al.*, 2003). Reduced GSH acts as an antioxidant and is involved directly in the reduction of most ROS generated during stress (Millar *et al.*, 2003; Foyer and Noctor, 2005; Shao *et al.*, 2008).

Transgenic plants exhibit greater heavy metal tolerance and accumulation of heavy metals in shoot mainly due to their enhanced chelation capacities by overexpressed enzymes involved in the biosynthesis of GSH, which is the precursor for PCs synthesis (Zhu *et al.*, 1999; Bennett *et al.*, 2003; Gisbert *et al.*, 2003; Tong *et al.*, 2004). MTs and PCs consist of amino acids and bind metals in thiolate complexes and thus demand a greater input of amino acids (especially Cys), sulfur and nitrogen from the plant as the level of accumulated metals rises (Tong *et al.*, 2004). Bonner *et al.* (2005) and Eapen and D'Souza (2005) stated that the gene, O-acetyl-L-serine (thiol) lyase (OAS-TL, At3g22460), forms a carbon skeleton for Cys biosynthesis, producing an amino acid which is a fundamental constituent of all metal chelators: MTs, PCs, GSH, and NA. Kawashima *et al.* (2004) stated that tobacco plants with altered levels of this protein in the cytosol and/or chloroplasts showed enhanced tolerance to Cd, Se, and Ni. Kawashima *et al.* (2004) reported markedly improved Ni tolerance (on medium with 500 μM Ni^{2+}, with 1.3 times higher biomass and 4.2 times longer roots) in *Nicotiana tabacum* overproducing OAS-TL from spinach *Spinacia oleracea*. Yadav (2010) stated that the concentrations of GSH, Cys, and OAS in shoot tissue are closely related to the ability to hyperaccumulate Ni in various *Thlaspi* hyperaccumulators collected from serpentine soils. Examples of such hyperaccumulators reported are *T. goesingense*, *Thlaspi oxyceras*, and *Thlaspi rosulare*, and non-accumulator relatives *Thlaspi perfoliatum*, *T. arvense*, and *A. thaliana* (Krämer *et al.*, 1997; Wenzel and Jockwer, 1999; Reeves and Baker, 2000; Guerinot and Salt, 2001; Peer *et al.*, 2003; Freeman *et al.*, 2004). High concentrations of OAS, Cys, and GSH in Austrian Ni hyperaccumulator *T. goesingense* coincide with high activity of both serine acetyltransferase (SAT) and GSH reductase (GR) enzymes. SAT catalyzes the acetylation of L-Ser to produce OAS. These changes in Cys and GSH metabolism are consistent with the ability of *T. goesingense* to hyperaccumulate Ni and resistance to oxidative stress (Peer *et al.*, 2003; Freeman *et al.*, 2004; Yadav, 2010).

For instance, in *T. goesigense*, the natural overproduction of GSH is considered a trait sustaining tolerance to oxidative stress caused by Cd and Ni (Boominathan and Doran, 2003a; Freeman and Salt, 2007). Besides,

overproduction of mitochondrial SAT encoded by TgSAT of *T. goesingense* promoted an accumulation of GSH in leaves of *A. thaliana*, providing increased resistance to Ni-induced growth inhibition and oxidative stress (Freeman and Salt, 2007). In addition, salicylic acid (SA) metabolites such as phenylalanine, cinnamic acid, salicyloyl-glucose, and catechol were also elevated in the hyperaccumulator *T. goesingense*, as compared to the non-accumulators *A. thaliana* and *T. arvense* (Freeman *et al.*, 2005a). Elevation in free SA levels of Arabidopsis, either through genetic modification or by exogenous feeding enhanced the specific activity of SAT. The increase in SAT activity raised GSH content and enhanced Ni resistance (Yadav, 2010).

The NA concentration can be increased by the overexpression of NA synthase (*NAS*) genes. For example, enhanced accumulation of Zn, Fe, Cu, Ni, and Mn in shoots of tobacco and the level of its tolerance to metals is induced by the transferred *NAS1* from *Hordeum vulgare* to tobacco (Takahashi *et al.*, 2003).

5.3.2.3. Proteins

Rascio and Navari-Izzo (2011) stated that the sequestration trait in plants relies, at least in part, on constitutive overexpression of genes that, in this case, encode proteins operating in heavy metal transfer across the tonoplast and/or plasma membrane and involved in excluding them from cytoplasm CDFs (MTPs), which mediate bivalent cation efflux from the cytosol, are important candidates for this process (Rascio and Navari-Izzo, 2011). Phylogenetic reconstruction has classified the majority of CDF family members into three groups, each containing characteristic members that share the same specificity toward the principally transported metal, i.e., Zn, Fe/Zn or Mn (Montanini *et al.*, 2007). MTP1, belonging to the Group I members of CDF family—Zn-CDFs, a gene encoding a protein localized at tonoplast, is highly overexpressed in the leaves of Zn/Ni hyperaccumulator plants (Dräger *et al.*, 2004; Kim *et al.*, 2004; Hammond *et al.*, 2006; Gustin *et al.*, 2009). Contrary to previous reports indicating plasma membrane localization (Kim *et al.*, 2004), Gustin *et al.* (2009) observed that TgMTP1 from *T. goesingense* localizes to the vacuolar membrane. TgMTP1 also was shown to be able to confer Ni, Cd, and Co tolerance when overexpressed in yeast (Persans *et al.*, 2001; Bloss *et al.*, 2002). As mentioned in the Section 5.3.2.1., high level expression of a vacuolar metal ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles (Persans *et al.*, 2001). Besides, RmCzcD from *Cupriavidus metallidurans*, belonging also to Zn-CDFs, is able to bind Zn, Co, Cu, and Ni (Anton *et al.*, 2004) and to alleviate Zn, Co, and Cd toxicity when overexpressed in bacteria (Anton *et al.*, 1999). In addition, WmFieF, belonging to the Group II members

of CDF family—Fe/Zn-CDFs from *C. metallidurans*, is mainly involved in Fe detoxification; it also mediates resistance against other divalent metals such as Zn, Co, Cd, and Ni, as shown by overexpression in *Escherichia coli* or in *C. metallidurans* metal sensitive mutants (Munkelt *et al.*, 2004).

NRAMP3 has been isolated from *T. caerulescens* (Oomen *et al.*, 2009; Wei *et al.*, 2009b) and its expression in roots is induced by Fe starvation and by the presence of Cd and Ni. In yeast, overexpression of TcNRAMP3 increased Cd content, enhanced Ni resistance, and reduced Ni accumulation, emphasizing the role of TcNRAMP3 in metal cations homeostasis rather than in metals accumulation (Wei *et al.*, 2009b; Maestri *et al.*, 2010).

Schulz and Kolukisaoglu (2006) reported the involvement of ABC transporters in a vast number of metabolic pathways and physiological processes, including hormone transport, plasma membrane channel regulation, and ion homeostasis. ABC transporters have been categorized based on structural similarities, in several subfamilies, however only two of these subfamilies, such as MRP and AMT/HTM, contain proteins involved in metal transport (Martinoia *et al.*, 2002). These contain two cytosolic, two large transmembrane regions and N-terminal extensions (Bovet *et al.*, 2003), and probably have a multifunctional role in plant homeostasis (Schulz and Kolukisaoglu, 2006; Zientara *et al.*, 2009). GUS has been used as a reporter to monitor the activity of the promoter of the AtMRP3 gene from *A. thaliana*, and then, the AtMRP3 promoter-GUS fusion expression cassette was introduced into the genome of two model plants, *A. thaliana* and *N. tabacum* (Zientara *et al.*, 2009). The results showed that this promoter can be used for driving the specific expression of genes involved in As, Cd, Ni, Co, or Pb metal phytoremediation, or for monitoring the presence of bioavailable As, Cd, Ni, Co, and Pb in the soil using transgenic reporter plants (Zientara *et al.*, 2009).

In *N. tabacum*, the plasma membrane localizes NtCBP4 (calmodulin binding protein) which is structurally similar to vertebrate and invertebrate K^+ and to nonselective cation channels (Arazi *et al.*, 1999). Overproduction of NtCBP4 in *N. tabaccum* exhibited increased uptake and translocation of Pb to shoots, reflected in the higher sensitivity of transgenes compared to controls (Arazi *et al.*, 1999). In contrast, hydroponically (by 70% longer roots on medium with 100 μM Ni^{2+}) grown *N. tabaccum* overexpression of NtCBP4 restricted the accumulation of Ni (shoot uptake of Ni reduced by 60% in plant grown in medium with 200 μM Ni^{2+}), thereby enhancing tolerance to elevated Ni concentrations (Bizily *et al.*, 1999).

Nickel and Co are ubiquitous and are often co-localized in natural environments (Gikas, 2008). When present in excess concentrations, both Ni and Co are expelled out of the bacterial cell to ensure detoxification (Blaha *et al.*, 2011). RcnA has been reported as an efflux pump responsible for

Ni and Co detoxification in *E. coli*. RcnA confers resistance to Ni and Co, and its expression is induced by these two metals and not by other divalent cations (Rodrigue *et al.*, 2005; Blaha *et al.*, 2011). Overexpression of MTs and GSH S-transferase fusion protein (GSM-MT) as well as nixA gene exhibits very high affinity for Ni in *E.* coli JM109 (Deng *et al.*, 2003).

6. Phytoremediation

6.1. Phytoextraction and Phytomining

Although there are many contaminated and/or Ni-rich soils in the world, unfortunately, modern mining technology cannot be economically applied to this ore, since a content of at least 30 g kg^{-1} Ni is required to proceed, which is never the case in the soils even if ultramafic rocks are present (Ni concentrations are between 1 and 7 g kg^{-1} of rock) (Li *et al.*, 2003b). Phytoremediation is emerging as a cost-effective method for *in situ* removal of the pollutants from matrices, and phytoextraction using hyperaccumulator plants is proving to be one of the most effective phytoremediation methods to clean up metal contaminated sites (Robinson *et al.*, 1999b; Nedelkoska and Doran, 2000; Chaney *et al.*, 2007).

The "bio-ore" produced by phytomining is much richer in Ni than the naturally occurring common Ni ores (Brooks *et al.*, 1998; Chaney *et al.*, 1998). The post-treatment methods of Ni hyperaccumulators include incineration followed by conventional smelting processes (Belevi and Moench, 2000; Keller *et al.*, 2005). Such metal recovery method is a more environmentally friendly approach than metal extraction through chemical leaching (Barbaroux *et al.*, 2009) and pyrolysis method (Koppolu and Clements, 2003; Boominathan *et al.*, 2004). There are evidences for extraction of high purity Ni from Ni-contaminated Alyssum biomass (Bani *et al.*, 2008; Chaney *et al.*, 2008). Additionally, recycling in metallurgy and using as Ni fertilizer to correct Ni deficiency in crops are both commercially viable options for the Ni recovered from the Ni-accumulated plant biomass (Mench *et al.*, 2010).

The hyperaccumulator plants for metal mining from sub-economic ore bodies have been reported previously. For instance, 1600 mg kg^{-1} Ni was measured in dried leaves of *H. floribundus*, grown in a Australian soil with Ni content of 700 mg kg^{-1} soil (Severne and Brooks, 1972) and excess of one percent Ni concentration was reported in *H. floribundus* (Cole, 1973).

The US Bureau of Mines conducted a Ni phytoremediation field study in 1995 at Reno, Nevada on serpentine soil containing Ni concentration of

3340 mg kg^{-1} and used naturally occurring strain of *S. polygaloides*, a Ni-hyperaccumulating member of the *Brassicaceae* family endemic to serpentine soils of California (Reeves *et al.*, 1981). At harvest the shoot Ni concentration of *S. polygaloides* averaged 5300 mg kg^{-1}, with a biomass yield of 4.8 Mg ha^{-1} (Nicks and Chambers, 1995). From this study it was concluded a net return of \$300–500 ha^{-1} yr^{-1} could be achieved assuming a Ni price of \$7.65 kg^{-1}, and a quarter of the energy of biomass combustion could be turned into electricity.

In Italy, Robinson *et al.* (1997a) used *A. bertolonii* as hyper Ni accumulator and were able to remove 72 kg Ni ha^{-1}, which was worth \$539.00. Brooks *et al.* (1999) reported that *B. coddii* is an excellent plant to use as hyperaccumulator for phytomining of Ni since it is perennial, has a high biomass, and tolerates severe climatic conditions such as frost and cool weather. With adequate fertilizer and soil moisture it is known to remove 100 kg ha^{-1} of Ni (Anderson *et al.*, 1999). Li *et al.* (2003b) reported that selected parental lines of *A. murale* and *Alysssum corsicum* had shoot Ni concentration as high as 22,000 mg kg^{-1} and biomass as high as 20,000 kg ha^{-1}.

However, Bennett *et al.* (1998) pointed out that phytoremediation and phytomining techniques are still in their infancy and will need to be proven over several years before there is complete scientific and commercial acceptance of their values. Although many Ni-hyperaccumulating species have been described, their remediation potential is greatly limited due to their low biomass and slow growth. Li *et al.* (2003b) concluded that in developing plants suitable for commercial phytoremediation, should take into account (1) identifying or creating ideal phytoextraction plants, (2) optimizing soil and crop management practices, and (3) developing methods for biomass processing and Ni extraction.

6.2. Optimizing Soil Management Practices

The solubility and availability of metals for plants may be affected by several soil factors such as pH, proportion of nutrient elements, soil organic matter content (SOM), cation exchange capacity (CEC), clay content, carbon exchange capacity, redox potential, and moisture, which determine the different uptake by different plant species and at different locations. Recently, Li *et al.* (2011) developed the quantitative relationships between Ni toxicity and Chinese soil properties (regression models between toxicity thresholds and soil pH, soil organic carbon content, and effective cation exchange capacity). These models showed good agreement with those developed previously by Rooney *et al.* (2007) for European soils ($R^2 = 0.87$), which are helpful to develop soil-specific guidance on Ni toxicity thresholds for China and Europe where majority of Ni-contaminated sites currently exist.

6.2.1. pH

Of the soil factors, pH is one of the most important parameters affecting absorption of metals by plants. Soil amendments have been used widely to alter the pH of the soil (Kukier and Chaney, 2001). In most cases, increasing soil pH results in a decrease of Ni concentrations in various plant species. Studies have shown that as Ni-contaminated soils are limed, or made calcareous, Ni uptake by various crops is significantly reduced (Crooke, 1956; Frank *et al.*, 1982; Bisessar, 1989; Cao *et al.*, 1993; Hooda and Alloway, 1996; Kukier and Chaney, 2001; Everhart *et al.*, 2006). A combination of lime stabilization to pH of 6 and the use of Agropyron as a revegetation species should provide a means to remediate and restore acidic soil contaminated with Ni up to a concentration of 100 mg kg^{-1} (Chen and Wong, 2006). Robinson *et al.* (1999a) reported the effect of $MgCO_3$, $CaCO_3$, and sulfur application on Ni and Co uptake by *B. coddii*. $MgCO_3$ increased soil pH from 6.9 to 8.7, resulting in a significant ($P < 0.05$) decrease in plant uptake of both Ni and Co. Sulfur application caused a decrease in soil pH from 6.9 to 5.5, hence increasing the plant uptake of both metals. Increasing soil pH was effective in ameliorating Ni phytotoxicity by converting soluble Ni into forms that are not available to the plants (Scheckel and Sparks, 2001; Sheoran *et al.*, 2009).

Management of soil pH and use of *Alyssum* spp. may facilitate the remediation of Ni-contaminated soils (Siebielec *et al.*, 2007). Nevertheless, Li *et al.* (2003a) raised a very important question regarding plant–soil–metal interactions: how much soil pH affects uptake of Ni and other metals by hyperaccumulator plants? They carried out both a greenhouse and a field experiment using the Ni hyperaccumulator species *A. murale* and *A. corsicum*. For the greenhouse experiment, Welland loam soil (Orthic Humic Gleysol) pH was adjusted using nitric acid and $CaCO_3$ to provide a range of pH before planting. For the field study, both Welland loam soil (Orthic Humic Gleysol) and Quarry muck soil (Terric Mesisol) pH was increased by limestone addition. The pH range of Welland loam soil was from 4.97 to 5.46, and the pH range of Quarry muck soil was from 5.4 to 6.32. An unexpected result of both experiments was that Ni uptake by these *Alyssum* species was reduced at lower soil pH and increased at higher soil pH (Li *et al.*, 2003a). Besides, Kukier *et al.* (2004) also carried out an experiment using *A. murale* and *A. corsicum* grown in Quarry muck (Terric Haplohemist) and Welland (Typic Epiaquoll) soils contaminated by a Ni refinery in Port Colborne, Ontario, Canada, and in the serpentine Brockman soil from Oregon, USA. Soils were acidified and limed to cover pH from strongly acidic to mildly alkaline. *Alyssum* grown in both industrially contaminated soils exhibited increased Ni concentration in shoots as soil pH increased despite a decrease in water-soluble soil Ni. A small decrease in *Alyssum*

shoot Ni concentration as soil pH increased was observed in the serpentine soil. Maximum Ni phytoextraction was achieved at pH 7.3, 7.7, and 6.4 in the Quarry, Welland, and Brockman soils, respectively. The differences in uptake pattern of Ni by Alyssum from different soils and pH were probably related to the differences in organic matter and iron contents of the soils. Moreover, Everhart *et al.* (2006) reported that the phytoextraction capability of *A. murale* increased as soil pH increased, which was not the case for *Alyssum sativa*. Further, Everhart *et al.* (2006) pointed out that the Ni specific bacterial biosensor was successful in predicting Ni bioavailability in the soils and suggested that higher Ni bioavailabilities occur in the soils at pH values of 5.1 and 6. These results are opposite to the response seen in crop plants and to the pattern of accumulation of other divalent metals (e.g., Mn and Zn) by these Ni-hyperaccumulator species. Li *et al.* (2003a) stated that further research is needed to elucidate the mechanisms responsible for the unusual pattern of Ni accumulation by *Alyssum* shoots in response to varying soil pH and other soil chemical properties that may influence Ni availability and growth of the hyperaccumulators.

6.2.2. Fertilizers

Serpentine soils are characterized by a pH of 6–8, low Ca and high Mg, and low levels of N, P, and K. The severely deficient levels of N, P, K, and Ca lead to most serpentine soils inhospitable to normal terrestrial plants (Nicks and Chambers, 1995; 1998). Addition of fertilizers such as N, P, K, and moderate amounts of Ca to soil, may not only support the growth of plants, but will also decrease the soil pH, hence increasing the metal bioavailability. Experiment with Ni hyperaccumulators grown in Ni-rich soil in Pojska, Albania, showed that shoot Ni concentrations ranging from 0.13% Ni in *Thlaspi ochroleucum* to 1.33% Ni in *A. murale* (Bani *et al.*, 2008). Fertilization of native *A. murale* with NPK promoted shoot biomass yields without affecting Ni concentration in shoot, resulting in increased Ni removal (Bani *et al.*, 2008). In fertilized and herbicide (anti-monocots)-treated plots the biomass yield progressively improved from 2.6 to 6.0 t ha^{-1}, and Ni removal increased from 22.6 to 69 kg ha^{-1} (Bani *et al.*, 2007; 2008). Kidd and Monterroso (2005) identified that increased uptake of Cr and Ni by *A. serpyllifolium* can be achieved by increasing plant biomass *via* liming and fertilization with N, P, and K. Naturally occurring populations of *A. bertolonii* in Italy were evaluated by Robinson *et al.* (1997b) for Ni accumulation. With the addition of fertilizers, the maximum annual biomass was approximately 300% of the control without reducing shoot Ni concentration. In another study similar trends were observed for *B. coddii* where addition of N increased Ni content from 2500 mg kg^{-1} to 4200 mg kg^{-1} in leaves (Robinson *et al.*, 1997a). Robinson *et al.* (1999a) further indicated that uptake of Co

and Ni by *B. coddii* was enhanced by sulfur fertilizer in a serpentine-spoil in New Zealand.

Higher levels of N-fertilization increased accumulations of Ni, Cd, and Zn along with biomass yield (Keller *et al.*, 2003, 2005; Keller and Hammer, 2004; Chaney *et al.*, 2008). Similarly, addition of ammonium sulfate to a soil doubled the concentrations of Zn and Ni in willow without affecting plant yield. Bennett *et al.* (1998) reported that N-fertilization increased the biomass of three hyperaccumulator species, *A. bertolonii*, *S. polygaloides* and *T. caerulescens*. In this study, *Alyssum* and *Thlaspi* had a slight reduction in Ni and Zn concentration when biomass of each plant was increased using an N fertilizer of 100 mg kg^{-1}. All of these studies indicate that management of soil fertility is vital for the development of successful commercial phytoextraction technology (Li *et al.*, 2003b; Sheoran *et al.*, 2009).

Moreover, studies showed that nutrient enrichment could influence both metal uptake and its distribution in the subcellular structures and macromolecular components of the phytoplankton. Nitrate addition enhanced the uptake of Ni by *Prorocentrum donghaiense*, whereas phosphate addition inhibited Ni uptake at high-Ni concentration. In addition, although the effects of nitrate concentration on Ni uptake were significant, they seemed relatively small in comparison with the effect of urea on Ni uptake under high-Ni treatment (Hong *et al.*, 2009). Nitrate or phosphate addition significantly affected Ni distribution in the subcellular structures and components. The majority of Ni was found in the soluble substances (>70%) and in the proteins (55.0—79.6%) of the algal cells. With an increase in nitrate concentration, the Ni content in the cell wall and soluble substances tended to decrease, but that in the organelles showed a tendency to increase. However, the increase of phosphate level increased the Ni content in the cell wall and soluble substances, while showing insignificant impact in the organelles (Hong *et al.*, 2009). Thus, the increasing nitrate concentration might cause the algal cells to suffer more from metal toxicity, but phosphate addition could show a protecting effect, since metal internalization in the cell wall could act on the protective mechanism and the organelle is usually sensitive to metal attack (Neumann *et al.*, 1995; Zenk, 1996; Hall, 2002; Hong *et al.*, 2009). Urea reduced Ni content in the amino acid-carbohydrate but elevated its content in proteins, and showed significant correlation with the protein content of the algal cells (Hong *et al.*, 2009).

6.2.3. Cation exchange capacity

Cations, such as Ca^{2+} and H^{+}, can influence Ni uptake and toxicity. A higher cation exchange capacity (CEC) could lead to more metal ions on the exchangeable sites. As discussed in Section 6.2.1., pH is an important soil property modulating the availability of Ni. Rooney *et al.* (2007) showed that exchangeable Ca concentration in soils is the best

predictor of the variation of toxicity threshold values (EC50 and EC10) of Ni for barley and tomato. Studies showed that Ca, H, and Ni competed for root binding sites of pea (*Pisum sativum* L.) with high pH and low Ca favoring more Ni accumulation (Wu *et al.*, 2010). At low pH, Ca accumulation is the key factor determining root growth of pea; while at medium to high pH, root elongation is more sensitive to Ni concentration (Wu *et al.*, 2010). Additionally, in hydroponic experiments, Weng *et al.* (2003) observed that increased pH led to increased Ni in oat roots.

6.2.4. Soil organic matter

Soil organic matter (SOM) can also have a significant impact on metal sequestration thus reducing the solubility of Ni by forming complexes with organic functional groups (Nachtegaal and Sparks, 2003). Soil organic matter strengthens soil buffering capacity, providing resistance against severe chemical changes. The ability of SOM to form complexes with metals depends on its high content of functional groups (carboxyl COOH, phenolic-OH, etc.) with COOH playing a predominant role in metal binding (Stevenson, 1994).

Soil organic matter plays an important role in ecosystem functioning, by improving physical and chemical properties of the soil and/or by buffering nutrient supply (Viventsova *et al.*, 2005). Cation exchange capacity of the soil is highly associated with clay minerals and SOM content of soil, and in sandy soils the CEC is rather low and predominantly controlled by the level of SOM (Smith *et al.*, 1993). Therefore, levels of exchangeable nutrient elements are generally higher in soils with a larger amount of SOM, since most nutrient elements are related to SOM, the maintenance of the SOM pool will ensure favorable nutrient conditions for ecosystem restoration.

In aquatic systems, dissolved organic matter (DOM) is known to reduce the bioavailability of Ni. Moreover, it was suggested that the concentration of dissolved organic carbon (DOC) plays a greater role than either DOC source or fraction in determining Ni speciation and subsequently the bioavailability and toxicity to *Hyalella azteca*, a common freshwater benthic invertebrate (Doig and Liber, 2006).

6.2.5. Soil moisture

Soil extractable Ni often decreased with increasing moisture content and in waterlogged soils, solubility of Ni is reduced considerably due to low redox potentials (Rieuwerts *et al.*, 1998) and formation of sparingly soluble sulfides (Marschner, 1995). Angle *et al.* (2003) stated that plant growth, exclusive of metal availability might be affected when hyperaccumulators are grown in high moisture soils. Overall, soil water plays a greater role in the amount of available Ni.

Angle *et al.* (2003) reported that extractable Ni in soil decreased with increasing soil water holding capacity (WHC) from 30 to 100%. Biomass of Ni hyperaccumulators (*A. murale* and *B. coddii*) and the Zn hyperaccumulators (*T. caerulescens*) was reduced at low soil moisture. Plants accumulated large amounts of metals from soil at higher soil moisture. Highest foliar concentrations of Zn or Ni were found at the two highest WHCs of 80 and 100%. In this study hyperaccumulators grow well under conditions of high soil moisture content and that they continued to hyperaccumulate metals.

6.2.6. Vegetation

The effect of other vegetation on the bioavailability of metals is uncertain. Pulford *et al.* (2002) showed that EDTA extractable Cd, Cu, Ni, and Zn in sewage sludge treated soil were higher under willow than in unplanted areas. On the other hand, Watson (2002) found evidence for depletion of extractable metals under the willow tree, as compared to concentrations in adjacent unplanted areas. Assessment of Ni bioavailability in smelter-contaminated soils of Port Colborne (Canada) indicated that large surface area of trees impact aerial deposits and elevated levels of Ni. The Ni level in the soil from a forest downwind of the refinery was approximately 22,000 mg kg^{-1}. However, field plots next to this forest-covered soil had approximately 5000 mg kg^{-1} of Ni (Everhart *et al.*, 2006).

6.2.7. Exogenous chelates

Exogenous chelates in soil have shown high effectiveness in enhancing phytoextraction of metals. Various reported chelates for Ni phytoextraction are the synthetic chelates such as EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), HEDTA (N-(2-hydroxyethy1)-ethylenediaminetetraacetic), NTA (nitrolenetriacetic acid), EDDS (ethylenediaminedisuccinic acid), and naturally occurring low-molecular-weight organic acids such as citric acid, His, gallic acid, and oxalic acid. Such substances are capable of forming complexes with metal ions, thereby modifying the bioavailability of heavy metals in soils.

Dramatic increases in the accumulation of Pb, Cd, Ni, Cu, and Zn in *B. juncea* in the presence of added EDTA and other synthetic chelants [DTPA, citric acid and CDTA (Trans-1, 2-cyclohexanediaminetetraacetic acid)] were reported by Blaylock *et al.* (1997). Soil applied EDTA is probably the most efficient chelate for increasing the concentrations of various metals in above-ground plant tissues (Cunningham and Ow, 1996; Blaylock *et al.*, 1997; Huang *et al.*, 1997; Vassil *et al.*, 1998). Its high efficiency relies on the strong chelating ability and also solubilization of poorly available metals in soils, followed by a largely passive accumulation of metal complexes in plant shoots through the transpiration stream (Blaylock, 2000; Sarret *et al.*,

2001). Chen and Cutright (2001) found that Ni was considerably mobilized and taken up by *H. annuus*, with increasing concentration of HEDTA (droxyethylethylenediaminetriacetic acid) (FeHEDTA) and EDTA. In particular, EDTA at a rate of 0.5 g kg^{-1} significantly increased the shoot concentrations of Ni from 15 to 117 mg kg^{-1}.

Metal uptake by *H. vulgare* and *H. annuus* in old animal waste storage lagoons, enriched with heavy metals, with and without the application of EDTA (0.5 g EDTA kg^{-1} soil) was investigated by Madrid and Kirkham (2002). The addition of EDTA did not increase heavy metal uptake by *H. annuus*, yet it induced higher Cu, Pb, and Ni accumulation in *H. vulgare* without reduction in biomass productivity. Meers *et al.* (2005b) repotted enhanced uptake of heavy metals (Cd, Cu, Ni, Pb and Zn) by *H. annuus* without a decrease in biomass productivity when EDTA (1, 3, and 5 mmol kg^{-1} soil) was applied shortly before harvest. Liphadzi and Kirkham (2006) showed that 1.0 g EDTA kg^{-1} applied to composted biosolids reduced Ni in the leaves and stems of *H. annuus*. However, when the applied EDTA levels increased to 2.0 g kg^{-1} compost, the concentrations of Ni in the roots increased to levels greater than the concentrations known to be toxic to plants (10 mg kg^{-1}) (Liphadzi and Kirkham, 2006). Therefore, in general, Ni in the composted biosolids tended to accumulate more in the leaves at low EDTA application rates of 1.0 g kg^{-1} compost or without EDTA. Additionally, it was reported that with the addition of 30 mmol kg^{-1} EDTA, the concentration of Ni in turfgrass *Lolium perenne* L. was increased 20 fold, as compared with the control (Duo *et al.*, 2005). The mobilization of Cr and Ni from an industrial soil was investigated using citric acid, His and EDTA. In this study, EDTA was the most effective for Ni mobilization, and a concentration of 0.05 mol L^{-1} EDTA allowed a good compromise between metal mobilization and preservation of the soil mineral integrity (Jean *et al.*, 2007). Besides, EDTA and citric acid were tested by Jean *et al.* (2008) to solubilize metals and to enhance their uptake by *D. innoxia*, the results showed that both the chelates increased the uptake of Ni, while EDTA was the most effective. For applications of 1 mmol kg^{-1} EDTA, the translocation factor (TF) of Ni was 6.7-fold higher than the control. However, when *B. coddii* was grown in substrates treated with 10 g kg^{-1} citric acid, 20 g kg^{-1} citric acid, 4 g kg^{-1} EDTA, or 10 g kg^{-1} citric acid and 4 g kg^{-1} EDTA, Robinson *et al.* (1997a) observed a decrease in shoot Ni concentration despite an increase in the concentration of soluble Ni in the rhizosphere. This loss was attributed to the competition with plant's own Ni-binding agents, thereby diffusing Ni downwards to the plant root system. Similar results were obtained in *B. coddii* by 0.5, 1, 2, and 4 g kg^{-1} NTA or DTPA for Ni but Co uptake was unaffected (Robinson *et al.*, 1999a).

Some synthetic chelating agents such as EDTA even allow plants, which are not considered as hyperaccumulators, to be usable for phytoremediation purposes, because chelates induce plants to take up more heavy metals than they normally accumulate (Liphadzi *et al.*, 2003). However, the slow degradation rate and long persistence of EDTA in soil can lead to uncontrolled leaching of metals, which may result in potential risks of surface and ground water pollution (Quartacci *et al.*, 2006). Uncontrolled use of EDTA creats limit for phytoextraction under field conditions (Lombi *et al.*, 2001; Wu *et al.*, 2004; Meers *et al.*, 2005a). Consequently, Evangelou *et al.* (2004) suggested using a combination of biodegradable chelators and a plant with a high biomass and adequate metal tolerance to increase phytoextraction efficiency.

Biodegradable chelate alternatives to EDTA such as EDDS and NTA, as well as the widespread natural sources-LMWOA (e.g., citric acid, His, gallic acid, oxalic acid) in greater public acceptance of phytoextraction technology since they are easily biodegraded and more environmentally compatible than synthetic chelators (Wu *et al.*, 2004; Meers *et al.*, 2005a; Quartacci *et al.*, 2005; Nowack *et al.*, 2006). However, NTA proved to be unsuccessful in enhancing plant uptake of heavy metals (Cd, Cu, Ni, Pb, and Zn) from a moderately contaminated calcareous soil by *Z. mays* (2 mmol kg^{-1} soil) (Meers *et al.*, 2004) or *H. annuus* (3.56 mmol kg^{-1} dry weight dredged sediment) (Meers *et al.*, 2005a).

Meers *et al.* (2005a) repoted that uptake of Cu, Zn, and Ni by *H. annuus* was higher in dredged sediment treated with EDDS than EDTA. Similar results have been reported for four different agronomic crops (*Brassica rapa, Cannabis sativa, H. annuus*, and *Z. mays*), cultivated on calcareous soils. EDDS induced more uptake of Cu and Ni than EDTA, while for uptake of Cd and Pb it was the other way around and Zn-uptake were similar between chelates (Meers *et al.*, 2005b). In woody species such as *Salix sp.*, EDDS enhanced the uptake of Cu and Ni (Meers *et al.*, 2007).

Turgut *et al.* (2004) found that Ni uptake in the leaves of *H. annuus* was 4.3-fold higher when treated with 3.0 g kg^{-1} citric acid, compared to 1.0 g kg^{-1} citric acid. Tatar *et al.* (1999) suggested that citric acid could be involved in Ni transport within cucumber plants. Singer *et al.* (2007) stated that exogenous His of 11.5 mmol kg^{-1} soil did not increase Ni phytoextraction, yet they did observe that the His-extractable fraction of soil Ni showed a high correlation with phytoextractable Ni. Moreover, do Nascimento *et al.* (2006) compared the performance of synthetic chelates (EDTA and DTPA) with LMWOA (citric acid and gallic acid) in enhancing phytoextraction of metals by *B. juncea* from multi-metal (Cd, Zn, Cu, and Ni) contaminated soils. They demonstrated the net removal of these metals caused by 10 mmol kg^{-1} citric acid or gallic acid can be as much as synthetic chelates at the same dose, without increasing

the risk of leaching for these metals. Such findings demonstrated that LMWOA can be as efficient as synthetic chelates for use in phytoextraction technology. A major reason for this is the lower phytotoxicity of LMWOA, especially gallic acid, to Indian mustard compared to EDTA. In addition, Hsiao *et al.* (2007) found that levels of Cr and Ni in the soil solutions, and the concentration and total uptake of Ni in shoots of *B. juncea* treated with LMWOAs (oxalic acid and citric acid) were lower than those treated with EDTA and DTPA. The difference in Ni hyperaccumulation between synthetic chelators and LMWOAs agreed with findings obtained by Wu *et al.* (2004) and Quartacci *et al.* (2005). But, the reduction of plant shoot biomass caused by the two synthetic chelators exceeds that caused by the LMWOAs. The low plant biomass did not assist phytoextraction by using EDTA and DTPA. Therefore, adding LMWOAs during phytoremediation can provide an environmentally compatible alternative, which may decrease the use of synthetic chelators.

It has been widely reported that the addition of exogenous chelators to growth medium has a significantly adverse effect on plant growth (Grčman *et al.*, 2001; Lai and Chen, 2005; Quartacci *et al.*, 2006). Tobacco (*N. tabacum*) showed toxic symptoms such as lower dry weight and chlorosis in growth medium applied with higher concentrations of LMWOA chelates. Evangelou *et al.* (2006) have reported significant toxic effects of citric acid at the concentration of 125 mmol kg^{-1} on *N. tabacum*. Application of NTA at 5 mmol kg^{-1} rate reduced the shoot dry weight of *B. juncea* significantly (Quartacci *et al.*, 2006). Soil application of 5 mmol kg^{-1} of EDTA and EDDS decreased shoots biomass by up to 60% and 52% for corn, and 76% and 61% for beans (Luo *et al.*, 2005). Application of HEDTA and EDTA reduced shoot biomass of *H. annuus* and increasing chelate application rates from 0.5 to 2 g kg^{-1} led to a severe yield reduction. Increasing growth medium concentrations of Cd, Cr, and Ni from 90 to 150 ppm along with increasing rates of these chelates reduced the average shoot biomass from 50% to 75% respectively (Chen and Cutright, 2001). Such reduction in plant growth was attributed to the combined application of heavy metals and chelates that exceeded the capacity of plants to activate defense systems, for instance, LMWOA may damage the plasma membranes which are normally stabilized by Ca and Zn ions (Kaszuba and Hunt, 1990). Synthetic chelating agents applied to growth medium at high concentrations are known to be toxic to plants (Cooper *et al.*, 1999; Navari-Izzo and Quartacci, 2001; Luo *et al.*, 2005). Sun *et al.* (2009) stated that, when chelate enhancement is used to improve photoextraction efficiency, other than the high metal uptake capacity and biodegradation property, a reasonable amount of the chelate addition should also be considered, to maintain high plant biomass.

6.2.8. Composts

Composts have also been added to soils to assist phytoremediation. In mine tailings restoration when clay loam soils were amended with composted biosolids, willows growing in such medium were very effective in phytoextraction of Mn, Cu, and Cd (Boyter *et al.*, 2009). Compost amendments (obtained using wasted tea leaves as the main carbon source and swine manure as the nitrogen source by mixing at a mass ratio of 20 to 1) are found to be effective in assisting the growth of rape seeds, sunflowers, tomatoes, and soapworts in silt loams, and in performing the phytoextraction of Cu, Ni, and Cr from water-washed silt loams (Sung *et al.*, 2011). Composts can not only supply nutrients to plants, but also can create loose and ventilated soils for plants growing in hostile soils. It is seen that both the CEC and the organic matter increase in the test soils after adding the compost (Sung *et al.*, 2011).

6.3. Biological Factors

The ability to accumulate metals varies between species and between ecotypes within a given species. For instance, Boyd *et al.* (2008) investigated the variation of Ni concentrations in *S. coronatus* populations growing on ultramafic soils and found that two populations hyperaccumulated Ni in leaves (means of 12,000 and 8800 mg kg^{-1}) whereas the others did not (means of 120 and 130 mg kg^{-1}). Besides, Boyd *et al.* (2002) reported mean leaf Ni concentrations of 12,100, and 680 mg kg^{-1} from two populations growing on ultramafic soils in Mpumalanga Province, South Africa. Boyd *et al.* (2004) investigated population-level variation in Ni concentrations of *B. coddii* on three sites (Groenvaly, Groenvaly Mine and Doyershoek) with similar Ni levels. Highest Ni levels in plants were found at Groenvaly (10,900 mg kg^{-1}) and Groenvaly Mine (10,700 mg kg^{-1}) sites and plant Ni concentrations were lowest at Doyershoek sites (8800 mg kg^{-1}) but still at hyperaccumulator levels. Therefore, selecting the most effective hiperaccumulator population can be valuable for the development of phytoremediation methods to clean up Ni-contaminated soils. In the past decade, many studies have focused on identifying or creating ideal phytoextraction plants and optimizing crop management practices (Li *et al.*, 2003b).

6.3.1. Microbe enhancement

As discussed in Section 5.1.1., using rhizosphere bacteria is an alternate way to enhance phytoextraction efficiency. Some bacteria are capable of entering the plant as endophytes that do not cause harm and could establish a mutualistic association (Hallmann *et al.*, 1997; Compant *et al.*, 2005). Though many reports attest to the role of rhizosphere microbial

assisted phytoremediation (Gentry *et al.*, 2004; Thompson *et al.*, 2005; Rajkumar *et al.*, 2006; Lebeau *et al.*, 2008), the bacterial endophytes offer several advantages over rhizosphere microbes (Rajkumar *et al.*, 2009). Endophytes are known to enhance plant growth and increase plant tolerance to heavy metal stresses by improving plant nitrogen fixation capacity, IAA production, phosphate solubilization and the production of a siderophores in rhizophere (Costa and Loper, 1994; Verma *et al.*, 2001; Muthukumarasamy *et al.*, 2002; Lee *et al.*, 2004; Wakelin *et al.*, 2004). Endophytes are known to improve plant growth and health by enhancing mineral nutrition, or increasing resistance or tolerance to biotic and abiotic stresses (Ryan *et al.*, 2008; Rajkumar *et al.*, 2009).

In addition to plant growth promoting potential, certain metal-resistant endophytes have been shown to be able to increase Ni accumulation in the plant. Idris *et al.* (2004) reported the siderophore production in Ni-resistant bacteria isolated from *T. goesingense* and pointed out that endophytes were able to tolerate larger concentrations of Ni than rhizosphere bacteria, indicating an adaptation of the endophytic microflora to the large heavy metal concentrations present in *T. goesingense* shoots. Barzanti *et al.* (2007) isolated 83 endophytic bacteria from roots, stems, and leaves of Ni hyperaccumulator plant *A. bertolonii*. *A. bertolonii* harbors endophytic bacteria shown to produce siderophores and promote the plant growth under Ni stress. However, in general the siderophore production among endophytes may be a general phenotype (Idris *et al.*, 2004; Sessitsch *et al.*, 2004) because endophytic lifestyle has to cope with extremely low levels of free iron ions in the plant tissue. Weyens *et al.* (2010) reported that inoculation of yellow lupine exposed to Ni and toluene with a Ni-resistant and toluene-degrading endophytic bacterium resulted in decreased Ni and toluene phytotoxicity, and reduced evapotranspiration of toluene. Yellow lupine inoculated with the endophyte *Burkholderia cepacia* VM1468 bacterium possessing the *ncc* (nickel–cadmium–cobalt resistance)-*nre* Ni resistance/sequestration system and the pTOM-Bu61 plasmid coding for constitutive trichloroethylene (TCE) degradation could result in a strong reduction of phytotoxicity for plants exposed to Ni and TCE and a significant increase in Ni uptake (Weyens *et al.*, 2010).

However, other studies showed that the presence of metal-resistant endophytes decreased the uptake of Ni by the plants. In order to improve phytoremediation of heavy metals, Lodewyckx *et al.* (2001) introduced Ni tolerance genes *ncc-nre* from *Ralstonia metallidurans* 31A into two endophyte strains (*B. cepacia* and *Herbaspirillum seropedicae*). When inoculated on *Lupinus luteus*, the Ni-resistant *B. cepacia* induced a significant increase in the concentration of Ni in root, but not in the shoot (Lodewyckx *et al.*, 2001; Rajkumar *et al.*, 2009). Similarly, the inoculation of Ni resistance *H. seropedicae* decreased Ni uptake in *L. perenne*; however, inoculation of

wild type strain showed similar effect indicating that the Ni resistance characteristics of endophytes are not responsible for the altered Ni uptake in plants (Lodewyckx *et al.*, 2001; Rajkumar *et al.*, 2009). Madhaiyan *et al.* (2007) reported that inoculation with methylotrophic bacteria, *Methylobacterium oryzae* and *Burkholderia sp.* (isolated from rice tissue), reduced Ni and Cd uptake in roots and shoots of tomato and also their availability in soil, hence, protecting tomato (*Lycopersicon esculentum*) seeds from the toxicity of high concentrations of Ni and Cd. Such effects were attributed to the bacterium ability to lower the level of ethylene stress induced by the Ni and Cd. Inoculation of endophytes may also contribute to the reduced phytotoxic effects of the metals by sharing the metal load through their ability of biosorption and bioaccumulation.

6.3.2. Cell engineering technology

Cell engineering technology is also of great significance for enhancing the phytoextraction efficiency. Gadd (2006) suggested that because polyploid plants are usually bigger in size and more active in transpiration, which is beneficial to the transportation of heavy metals in root-to-shoot process. Crosses were made between *B. juncea*, a high biomass Pb accumulator species and *T. caerulescens*, a known Zn and Ni hyperaccumulator to produce somatic cell hybrids, both symmetric and asymmetric hybrids were bigger in size than both the parents and also had increased resistance and better hyperextraction of Pb, Ni, and Zn (Gleba *et al.*, 1999; Zhao, *et al.*, 1999; Dushenkov *et al.*, 2002). Eapen and D'Souza (2005) concluded that it appears to be worthwhile attempting the somatic cell hybridizations between high biomass candidate plants and low biomass but higher metal hyperaccumulators to develop hybrids with high biomass and enhanced hyperaccumulation capabilities.

6.3.3. Genetic engineering

Using genetic engineering to combine the desirable characteristics of hyperaccumulator genotypes is of great power in improving phytoremediation. Genetically diverse Ni-hyperaccumulator species and ecotypes of Alyssum were collected and preliminary genetic screening experiments were conducted to identify Ni-hyperaccumulator species and ecotypes suitable for further genetic improvement (Li *et al.*, 2003b). A field experiment was carried out in Cave Junction, Oregon to assess their genetic potentials and from this study six Ni-hyperaccumulator species were selected, including 125 *A. murale* and 45 *A. corsicum* accessions. Mean shoot Ni concentrations among *A. murale* and *A. corsicum* ranged from 4200 to 20,400 mg kg^{-1}. This finding indicates that genetic parental lines can be selected for breeding of improved phytoextraction cultivars (Li *et al.*, 2003b). Identification of plants with superior genetic traits for high metal uptake, larger biomass production, and superior

tolerance to soil heavy metal content will help advance phytoremediation technologies, and obtain plant types that are superior hyperaccumulators with high biomass and tolerance to toxic levels of heavy metals. These superior plant types may also provide genetic material capable of being introduced into other species (Robinson, 1997; Sheoran *et al.*, 2009). However, development of food crops with genetic modifications to serve as hyperaccumulators is also of concern as metals in products from such crops might get entry into food chain.

Transgenic approaches successfully employed to promote phytoextraction of Ni from soil by their accumulation in the above-ground biomass involve several aspects including: (a) implementing metal transporters along with modulation of the specificity of the metal uptake system, (b) improving the production of metabolic enzymes and biochemically transform metals to less toxic forms, (c) enhancing the production of intracellular ligands and the efficiency of ions' entrance into cells, and (d) facilitating the translocation in xylem or other part (Kotrba *et al.*, 2009; Wu *et al.*, 2010).

With the powerful tool of genetic engineering, many Ni-related genes have been introduced into plant cells and have effectively enhanced the phytoremediation of Ni from soil (as described in Section 5). Besides the genetically engineered plants, the genetically engineered bacteria also have shown many advantages in Ni remediation (Fulkerson *et al.*, 1998a, 1998b; Lopez *et al.*, 2002; Deng *et al.*, 2005; Freeman *et al.*, 2005b). However, the potential risks of the application of genetic engineering and low public acceptability, especially the ecological and environmental risks and regulatory constraints for testing and application in the field of genetically modified plants is of a great concern (Shukla *et al.*, 2010). Kotrba *et al.* (2009) pointed out that genes from genetically modified cultivated plants may transfer to wild relatives, thereby posing risks. The possibility of transformations of the natural flora by cross-pollination over long distances, the risk of invasion of crop plants, and the potential loss of diversity should be taken into serious consideration. Possible potential negative impacts of genetically engineered plants on the soil and rhizophere microorganisms, herbivores and other organisms along with their impact on food chain should be carefully evaluated. Linacre *et al.* (2003) suggested that it is essential to do risk assessment scenario for transgenic projects. Further, he stated that the risks of transmission of these genetically engineered traits are low, because such plants would be in isolated industrial districts, rather than in agricultural areas. Harvesting plants used for phytoextraction before flowering could substantially reduce the threat of crossing with the relatives and uncontrolled spreading of pollen or seeds. Krämer (2010) has extensively discussed the gentic and molecular basis of metal hyperaccumulation for Zn and Cd, and some of the approaches given

here could be useful for Ni. Detailed discussion on this area is beyond the scope of this review.

A thorough risk assessment study should be performed prior to the use of genetically engineered plants for phytoremediation. Excellent publications are available to provide needed guidance in risk assessments of transgenic plants (Wolfenbarger and Phifer 2000; Ervin *et al.*, 2000; Pilon-Smits and Pilon, 2002; Linacre *et al.*, 2003).

6.3.4. Plant management

Optimum plant management measures may effectively shorten the phytoremediation cycle. For instance, weed control chemicals are used to reduce weed competition with crops for soil nutrients, moisture, and light (Li *et al.*, 2003b). Herbicide application before harvest, improved the removal of Cr, Ni, and especially Zn by two to 3-fold in comparison to control plants due to enhanced transpiration by crops (Claus *et al.*, 2007). Li *et al.* (2003b) have conducted greenhouse and field tests of twelve herbicides for use with *A. murale* and *A. corsicum*, evaluating dose, mode of delivery, and use in combination. Further, they developed a strategy of weed control that used a combination of chemicals together with some mechanical control measures.

Besides, population density (plants ha^{-1}) is expected to be an important management variable, the optimal planting density for commercial production is affected by soil physical properties, organic matter and fertility levels, hyperaccumulator species, and other factors. For *A. murale* and *A. corsicum*, on average, the optimal density is one plant per 0.25 m^2 (Li *et al.*, 2003b). Under laboratory conditions, *Lemna gibba* (Lemnaceae) were cultured in vertical cylinders pot (height: 15 cm, diameter: 16.2 cm), which were filled with liquid nutrients. The results showed that at high population densities (800 or 1600 seedlings pot^{-1}) the growth rate of *L. gibba* decreased with increasing density. Especially, at a density of 1600 seedlings pot^{-1}, the net growth rate became negative. This lower growth rate may be related to lower local temperatures and nutrient supply within *L. gibba* mats. Additionally, high population densities caused decreased Ni accumulation by *L. gibba* (Demirezen *et al.*, 2007).

Adapting transplanting method of cultivation can shorten the phytoextraction cycle (Wei *et al.*, 2008). It may be most economical to establish perennial cropping systems for phytoremediation where crops are harvested every year without the cost of repeated yearly seeding. In some cropping systems it may be more profitable to reseed each year or every other year to maintain adequate plant desnsity. Li *et al.* (2003b) concluded that these choices could depend on the seasonal rainfall or availability of irrigation at the production locations. Experiments may need to be conducted in different regions of the world under different ecosystems to evaluate whether annual, biennial, or perennial crop

management systems are used and the adapted phytoremediation techniques are efficient and cost-effective.

7. Conclusions

An understanding of the mechanisms of Ni uptake and hyperaccumulation by plants has been considerably advanced in recent years. The pump system such as PM H^+-ATPase, Ni^{2+}/H^+ antiport system, V-ATPase, and V-PPase, the chelators such as His, NA, MA, citrate, malate, MTs, PCs, SA and its metabolites, and antioxidation-related genes GSH, Cys, and OAS, and transport proteins such as TjZnt1, TjZnt2, TjNRAMP4, HMA4, TgMTP1, RmCzcD, WmFieF, TcNRAMP3, AtMRP3, and NtCBP4 are all reported to have an effect on Ni uptake, transport, and/or detoxification in plant. The spatial distribution and particularly the cellular localization of Ni in the plant tissues may help improve understanding of the physiological effects of Ni on plants. All of these developments are of great importance for comprehending the physiological processes of Ni movement from soil to plant's cellular compartments, and hence, applying the plants for the clean up of Ni-contaminated soils may become a reality. However, most previous experiments were conducted at a laboratory scale and in relatively controlled conditions for a short period of time. The slow growth and small biomass of Ni-hyperaccumulator plants, have largely restricted their effectiveness for phytoremediation in the field. Therefore, although Ni is a metal of high value, few commercial phytomining projects have been demonstrated.

Combination of the phytoremediation process with modification of soil physical, chemical, microbiological properties, and other biological means such as cell and genetic engineering, may significantly shorten the time required to complete the entire remediation process. Although genetic engineering poses potential ecological and environmental risks, its potential to improve phytoremediation should be further explored. Rhizosphere microbes especially the PGPR, endophytic bacteria and end-ectotypes mycorrhizae can effectively assist in phytoremediation by lowering soil pH, producing compounds such as antibiotics and antifungals, organic acids, hormones (e.g., IAA), enzymes, siderophore, and metal-chelating agents, enhancing nitrogen fixation, and providing nutrients to the plant. Application of fertilizers such as N, P, K, Ca, and S, composts, and some exogenous chelates such as EDTA, DTPA, HEDTA, NTA, EDDS, citric acid, His, gallic acid and oxalic acid may facilitate the phytoremediation. Therefore, further integrated multidisciplinary research efforts that combine plant biology, genetic engineering, soil

chemistry, microbiology, and agronomy, as well as agricultural and environmental engineering are required.

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