Chapter 3

Coping Mechanisms of Plants to Metal Contaminated Soil

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Additional information is available at the end of the chapter

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1. Introduction

Metals such as cobalt (Co), copper (Cu), iron (Fe) and nickel (Ni) are essential for normal plant growth and development since they contribute to the function of many enzymes and proteins. However, metals can potentially become toxic to plants when they are present at high levels in their bioavailable forms (Hall, 2002). Phytotoxic levels of one or more inorganic ions in soil can be found in various parts of the world. These toxic sites occurred through natural processes or by anthropogenic effects. Naturally toxic soils include saline, acidic and serpentine soils, while anthropogenic polluted soils occur through mining activities, aerial fallout, and the run-off from galvanized sources of electricity pylons or motorway verges polluted by vehicle exhaust fumes (Bradshaw, 1984). The biochemical effect of metals on plants varies and the excess metal usually results in oxidative damage which affects their phenotype (Kachout *et al.*, 2009)

Plants colonizing metal-contaminated soils are classified as resistant and have adapted to this stressed environment. Heavy metal resistance can be achieved by avoidance and/or tolerance. Avoiders are plants that are able to protect themselves by preventing metal ions from entering their cellular cytoplasm, while tolerant plants are able to detoxify metal ions that have crossed the plasma membrane or internal organelle biomembranes (Millaleo *et al.*, 2010). Based on strategies used by plants growing on metal-contaminated soils, Baker and Walker (1990) classified them into three categories; metal excluder, indicators and accumulators/hyperaccumulators. The excluder group includes the majority of plant species that limit the translocation of heavy metals and maintain low levels of contaminants in their aerial tissues over an extensive range of soil concentrations. Plants that are metal indicators accumulate metals in their harvestable biomass and these levels generally are reflective of the metal concentration

in the soil. Metal accumulators/hyperaccumulators are plants that increase internal sequestration, translocation and accumulation of metals in their harvestable biomass to levels that far exceed those found in the soil (Mganga *et al.,* 2011; Baker and Walker 1990). Plants can accumulate and cope with the effects of high internal metal concentrations by the upregulation of the antioxidant defense system. This system is activated in order to respond to the deleterious effects caused by reactive oxygen species (Solanki and Dhankhar 2011).

Coping strategies allow the establishment of plant communities on metal contaminated soils. This is possible since some plants have adapted to these hostile sites by evolving mechanisms to deal with the toxic effects of metals in soil on plants. There is a need of identifying plants that are able to deal with excess metal in soil. Without these plants, the lands would remain barren and unsustainable.

The importance of plants in the remediation of heavy metal polluted soil is discussed in details in the present chapter. A review of the current knowledge on metal resistance mechanisms, as well as the potential genes and their role in metal homeostasis in plants will be examined. Finally, the coping mechanisms used by plants growing under metal contamination will be discussed.

2. Remediation of heavy metal contaminated sites

Soils that are heavily contaminated by metals may pose health risks to humans and to other living organisms in an ecosystem. Current techniques used to remediate metal contaminated soils include excavation, chemical stabilization, soil washing or soil flushing, but these methods are costly and impractical. There is a need to develop effective, low-cost and sustainable methods for soil bioremediation. The revegetation of these sites appears to be the most suitable method for long term land reclamation since plants can improve nutrient soil conditions. This can lead to the establishment of a self-sustaining vegetative cover, which in turn can prevent soil erosion (Wei *et al.,* 2005). Phytoremediation is an inexpensive and solar-driven approach that is performed *in situ*. It can be used to remove, stabilize and detoxify organic and inorganic pollutants including heavy metals from air, soil and liquid substrates (Salt *et al.,* 1998). An example of a reclaimed metal contaminated site in the mining region of Northern Ontario (Canada) is illustrated in figure 1. Plant species selected for land reclamation should grow and spread fast and be able to establish an effective soil cover. It is therefore important to search for plants that have spontaneously colonized these disturbed sites. Moreover, heavy metal contaminated mining sites exhibit physiochemical characteristics that are not suitable for the vast majority of plant species; hence the colonization of these sites is slow. However, plants that are resistant to this toxic environment can easily spread since there is a lack of competitors. It has been demonstrated that annual species have an extensive adaptive capacity compared to perennial genotypes due to their long-term natural selection (Wei *et al.,* 2005).

Phytoremediation is composed of five main subgroups: phytoextraction, phytovolatilisation phytostabilization, phytodegradation and rhizofiltration. Phytoextraction is a process by

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which plants extract metals from soil by accumulating them in their aerial biomass. These plants can be harvested and metals can be extracted from their tissues. Plants that accumulate metals in their aerial tissues have been involved in the phytoextraction of several metals including Cd, Cr, Cu, Hg, Pb, Ni Se and Zn (Yong and Ma 2002).

The accumulation of metals by plants is interesting from an environmental or agronomic point of view. In mining or industrial sites, as well as their surrounding areas, heavy metals are responsible for severe soil contamination. In these cases, accumulator plants could be used for phytoremediation as they are likely able to remove metals from soils (Salt *et al.*, 1998; Salt *et al.*, 1995). Since some heavy metals are also essential minerals that can be deficient in staple food crops, genetic determinants of hyperaccumulation could be utilized in biofortification to improve the nutritional value of these crops (Frérot *et al.*, 2010; Cakmak, 2008; Jeong and Guerinot 2008; Mayer *et al.*, 2008).

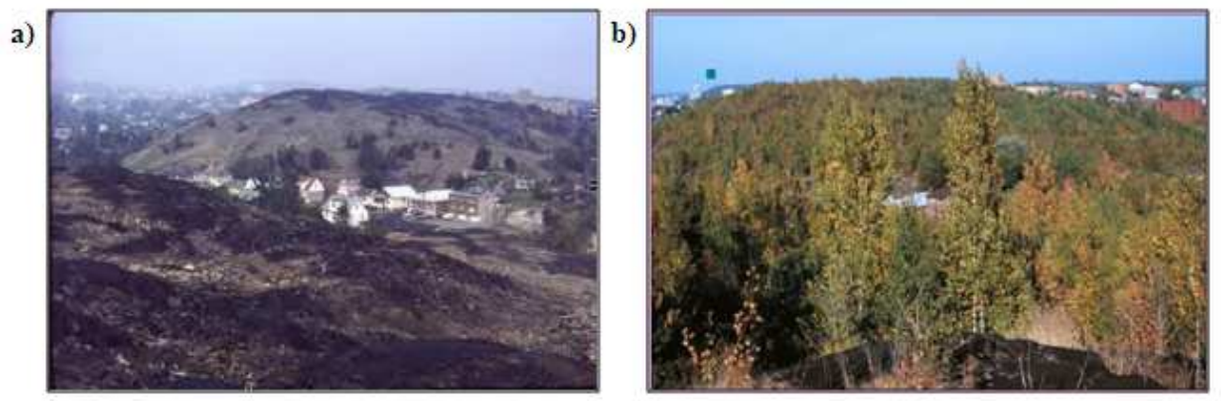

Figure 1. A metal contaminated site in Sudbury, Ontario, Canada; a) before remediation and b) after remediation (photos courtesy of Keith Winterhalder and David Pearson from Laurentian University).

These metal accumulator plants could also convert metals and release them in a volatile form. This process is known as phytovolatilization. Phytostabilization on the other hand, is a method that uses plants with a low ability for metal uptake to stabilize the contaminated soil thus preventing erosion. This limits the metals from entering the food chain. Plants can also be utilized for phytodegradation since they can in combination with microorganisms degrade organic pollutants. Finally, rhizofiltration is a process by which plant roots absorb metals from waste streams (Pulford and Watson 2003; Dushenkov *et al.*, 1995).

3. Resistance

Levitt (1980) stated that heavy metal contaminated environments act as stress factors on plants, which causes physiological reaction change that reduces or inhibits plant vigor and growth.

A plant showing injury or death due to metal stress is deemed sensitive to its environment. On the other hand, resistant plants can survive and reproduce under metal stress conditions (Ernst *et al.,* 2008). In general, plants can achieve resistance to heavy metals by avoidance or tolerance.

3.1. Avoidance

Avoidance occurs when plants restrict the uptake of metals within root tissue by several strategies. In environments where the soil metal contamination is heterogeneously distributed, plants can prevent metal uptake by exploring less contaminated soil. Another avoidance strategy involves mycorrhizal fungi, where they can extend their hyphae outside the plants rooting zone up to several tens of meters and transfer the necessary elements to the plant (Ernst, 2006; Baker, 1987). Also, these metal tolerant fungi can increase plant metal resistance by changing the metals speciation or by restricting the metal transfer into the plant (Ernst, 2006). Arines *et al.* (1989) found that mycorrhizal *Trifolium pratense* (red clover) plants growing in acid soils had lower levels of Mn in their roots and shoots as compared to the non-mycorrhizal plants. Plants can also restrict contaminant uptake in root tissues by immobilizing metals for example through root exudates in the rhizosphere. A role of root exudates is to chelate metals and stop their entry inside the cell. The cell wall has also been found to be involved in restricting metal uptake into the cell's cytoplasm (Mganga *et al.,* 2011).

3.2. Tolerance

In the absence of avoidance strategies, some plants can grow and survive in soil contaminated with toxic levels of heavy metals which are otherwise lethal or detrimental for growth and survival of others genotypes of the same or of different species (Maestri and Marmiroli 2012). Plants exhibiting tolerance are internally protected from the stress of metals that have entered the cell's cytoplasm (Baker, 1987). Metallophytes (metal tolerant plant) can function normally even in the presence of higher plant-internal metal levels. Plants adapt to their environments by developing heritable tolerance mechanisms. Tolerance to specific metals has evolved independently several times in different species from local non tolerant ancestral plant populations (Schat *et al.,* 2000). Plants can exhibit tolerance to metals that are present in surplus in the soil. Each metal is under control of specific genes.

According to Bradshaw (1991) most species are in a state of genostasis. It is the restriction of genetic variability which limits the evolution of the population/species. In the absence of avoidance pathways, metal contaminated soil acts as a selection force on a population, where only the plants with tolerant genotype can survive and reproduce. This leads to a bottleneck, where few individuals survive and reproduce. In turn, metal tolerant populations can evolve rapidly following a disturbance such as contamination of soil with heavy metals. Plant adaptation to these sites occurs in populations for which tolerance variability already exists prior to the contamination (Maestri and Marmiroli 2012; Baker, 1987). Genes for the tolerance of metals are pre-existing at a low frequency in non tolerant populations of certain plant species (Ernst, 2006; Macnair, 1987).

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3.2.1. Variation in tolerance and accumulation characteristics

Variation occurs between species, populations and clones for tolerance and accumulation of metals. Assunçãno *et al.* (2003) found differences in the degree of chlorosis and concentration of metal for *Thlaspi caerulescens* (currently named *Noccaea caerulescens*) populations when grown in hydroponic solutions containing various Ni, Cd and Zn concentrations. Visioli *et al.* (2010) found differences in growth, morphology and Ni accumulation capacity when the Ni hyperaccumulator *T. caerulescens* and the non-metal adapted *T. caerluescens* were exposed to different Ni concentrations in hydroponic solutions. Besnard *et al.* (2009) used cleaved amplified polymorphic sites (CAPs) and microsatellites to determine the genetic variation for *T. caerulescens* populations from metalliferous and non-metalliferous sites from Switzerland. They found a correlation between the level of heavy metals in soil and the variation at the target loci for the genes involved in encoding metal transporters. Basic *et al.* (2006) found similar results when they analyzed the genetic variation of different *T. caerulescens* population sampled from different soil types with single nucleotide polymorphism (SNPs) in target and non target genes. These results were also observed in *Populus spp.* Marmiroli *et al.* (2011) compared *Populus* clones and found variation in their capacity to accumulate Cd. This variation in Cd accumulation between clones was correlated with SNPs at some target genes. These results imply that gene flow is limited between individuals found on metal contaminated and those from uncontaminated sites, at least for the loci that are involved in the fitness of the individuals (Visioli *et al.,* 2012). On metal-enriched soil, there is a strong selection of local offspring, which conserves the metal tolerant genotypes (Ernst, 2006).

3.2.2. Genetics of tolerance to metals

Identifying genes involved in a specific adaptation is challenging. Metal tolerance and accumulation in plants are complex genetic systems. Plants have to modify their physiological processes in order to be able to survive in the environment in which they have germinated. In turn, the survival of a population to the contaminated environment is dependent on the inheritance of favourable traits. Tolerance mechanisms are heritable and variable, resulting from genes and gene products (Maestri and Marmiroli 2012). Variation in the evolution of metal tolerance exists over species, populations and clones (Baker, 1987). Some species do not show variation in tolerance and accumulations. In order to determine genes involved in metal tolerance and accumulation, segregating analyses were used, where parents with contrasting phenotypes were crossed to produce progeny. Studies have determined that in many species, metal tolerance and accumulation are genetically independent (Assunçãno *et al.,* 2006). For example, in *Arabidopsis halleri*, Cd tolerance and accumulation segregated as independent traits while Cd and Zn tolerance and accumulation cosegretated. In this later species, two or more genes were proposed to be involved in Cd and Zn accumulation but only one gene for Cd and Zn tolerance (Bert *et al.,* 2003; Bert *et al.,* 2002). In *T. caerulescens*, no genes involved in Cd, Zn and Ni tolerance and accumulation cosegregated. This suggests that there is a high probability that the genetic and physiological mechanisms for these traits are distinct from each other (Yang *et al.,* 2005a; Maestri *et al.,* 2010; Richau and Schat 2009; Assunçãno *et al.,* 2006; Zha *et al.,* 2004). As a result, it is not possible to conclude that a plant with high levels of metals in

aerial biomass is also metal tolerant. The concentration of metals in above ground tissue serves as an indication of the plant's potential metal tolerance (Frérot *et al.,* 2010).

Several techniques have been used to isolate and identify genes involved in heavy metal tolerance in plants, one of which is the quantitative trait loci (QTL) mapping. QTL mapping is a powerful tool in examining complex adaptive traits and in determining the number of genes involved in a trait as well as the genes effects and their interactions (Willems *et al.,* 2007). By mapping QTLs, it can be possible to identify or validate candidate genes involved in a complex trait such as metal tolerance and accumulation (Willems *et al.,* 2007). Other techniques used to identify genes for metal tolerance and accumulation are functional complementation in yeast mutants defective in metal homeostasis. These methods use plant cDNA expression libraries, as well as the identification of hypothesized pathways based on sequence similarities with plant cDNA libraries and genomic sequences (Lal, 2010). Transcriptome analyses have also been used to reveal genes involved in hyperaccumulation by analysing the differences in expression profiles or regulation-level of hyperaccumulator and non hyperaccumulator plants (Colzi *et al.,* 2011).

Few specific major genes have been found for Cd, Cu, Ni and Zn tolerance in *Silene vulgaris* by crossing plants from a metalliferous site with non tolerant plant from a nonmetalliferous site (Schat *et al.,* 1996; Schat *et al.,* 1993). Similar results were reported for Cu tolerance for *Mimulus guttatus* (Macnair, 1993) and Zn tolerance for *Arabidipsis halleri* (Bert *et al.,* 2003). In *S. vulgaris* and *M. guttatus*, modifier genes (minor genes) were involved in Cu tolerance, thus increasing tolerance and enhancing the effect of the major gene(s) (Smith and Macnair 1998). Only two QTL were involved in Ni accumulation and tolerance in *S. vulgaris* (Bratteler, 2005).

Studies aiming at identifying associations between molecular markers and metal tolerance and accumulation trait have been performed using interspecific and intraspecific crosses. When a high Zn accumulating *T. caerulescens* parent was crossed with a low Zn accumulating parent, two major QTLs were found to be involved in the increased of Zn accumulation in root (Assunçãno *et al.,* 2006). Deniau *et al.,* (2006) performed QTL mapping for the hyperaccumulation of Zn and Cd in *T. caerulescens*. They found two QTLs responsible for Cd and two for Zn accumulation in roots. In addition, one QTL for Cd and three QTLs for Zn accumulation in shoot were characterized. Macnair *et al.* (1999) reported a major gene involved in Zn tolerance from the analysis of F_2 progeny derived from a cross between *A. halleri* (tolerant parent) and *Arabidopsis lyrata* (sensitive parent). Willems *et al.* (2007) generated a backcross progeny from the interspecific cross between *A. halleri* (tolerant parent) and *A. lyrata (*sensitive parent) and identified three major additive QTLs involved in Zn tolerance in *A. halleri*. These QTLs were mapped to three different chromosomes (3, 4 and 6) and colocalized with genes that have been known to be involved in metal tolerance and accumulation. *HMA4* (*Heavy Metal ATPase 4)* encodes a P-type ATPase pump localized at the plasmamembrane involved in loading Zn and Cd into the xylem. *MTP1*-A and *MTP1-B* are Metal Tolerance Protein- vacuolar transporters that are involved in Zn tolerance) (Gustin *et al.,* 2009; Krämer, 2005). Three new QTLs were identified and mapped to chromosomes 4, 6 and 7 by Filatov *et al.* 2007, when F_2 progenies from a similar interspecific cross were analyzed. Frérot *et al.* (2010) also found Zn accumulation to be polygenic using *A. halleri* X *A. lyrata petraea* progenies. They determined

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that Zn accumulation is controlled by two QTLs in low Zn concentration and three QTLs in high Zn concentration. Four of the five QTLs mapped for Zn accumulation in their study were also reported in previous studies using *A. halleri* X *A. lyrata petraea* progenies (Frérot *et al.,* 2010; Filatov *et al.,* 2007; Filatov *et al.,* 2006).

Courbot *et al.* (2007), also using progeny from interspecific cross between *A. halleri* and *A. lyarata* determined three QTLs involved in Cd tolerance. A major QTL region was found to be common to Cd (Courbot et al 2007) and Zn (Willems et al 2007) tolerance and was colocalized with the *HMA4* gene. Hanikenne et al (2008) identified the role of *HMA4* using RNAi-mediated silencing. They reported that when the expression of *HMA4* was down-regulated, less Zn was translocated from the root to the shoot. When this gene was expressed in *A. thaliana*, an increase in Zn translocation to aerial tissue was observed. This increase in Zn translocation in *A. thaliana* plants resulted in signs of Zn hypersensitivity. Therefore, the expression of *AhHMA4* alone was not adequate for Zn detoxification. Additional genes are involved in the *A. halleri* Zn hyperaccumulation (Hanikenne and Nouet 2011; Frérot *et al.,* 2010).

Using segregating progeny resulting from intraspecific crosses between a high Cd accumulating parent and a low Cd accumulating parent for *Glycine max*, Benitez *et al.* (2010) identified a major QTL in seeds that was named cd1. This gene was mapped on chromosome 9. Jegadeesan *et al.* (2010) also identified a major QTL, *cda1*, associated with Cd accumulation in seeds of *G. max*. These two major QTLs were mapped to the same region of chromosome 9 which suggested that cd1 and *cda1* may be identical. Both QTLs were found to be a dominant major gene involved in the control of low Cd uptake. By analyzing the *G. max* genome, Benitez *et al.* (2010) revealed that the cd1 QTL is localized in the vicinity of the P_{1B}-ATPase gene (designated as *GmHMA1)* and proposed that this gene is involved in the transport of Cd. Benitez *et al.* (2012) found a single-base substitution between two cultivars, Harosoy (high Cd content in seed Cd) and Fukuyutaka (low Cd content in seed) in this P_{1B}–ATPase gene. This mutation resulted in an amino acid substitution (glycine in Fukuyutaka and glutamic acid in Harosoy) in GmHMA1a. Since the glycine residue at the amino acid substitution site was conserved in AtHMA3, AtHMA4, AtHMA6 and AtHMA7, it was suggested that the GmHMA1a from Fukuyutaka was the wild type, responsible for low Cd accumulation in seed (Benitez *et al.,* 2012). A dominant major gene involved in the control of Cd uptake was also observed in wheat (*Triticum aestivum*) (Clarke *et al.,* 1997) and oat (*Avena sativa* L.) (Tanhuanpää *et al.,* 2007). QTL analyses have also been performed in radish (*Raphanus sativus* L.). Xu *et al.* (2012) found a major QTL and three minor QTLs responsible for Cd accumulation in radish roots which were mapped on linkage groups 1, 4, 6 and 9. Induri *et al.* (2012) identified major QTLs for Cd response in *Populus* by performing a pseudo-backcross pedigree of *Populus trichocarpa* Torr. & Gray and *Populus deltoides* Bart. These QTLs were mapped to two different linkage groups. They performed a whole-genome microarray study and they were able to identify nine Cd responsive genes, which included a metal transporter, putative transcription factor and an NHL repeat membrane-spanning protein. Additional candidate genes located in the QTL intervals included a glutathione-S-transferase and putative homolog of a glutamine cysteine ligase.

Several QTL studies on rice (*Oryza sativa* L.) have been conducted to determine the number of genes involved in metal accumulation and tolerance. Three putative QTLs involved in Cd accumulation have been found on chromosomes 3, 6 and 8 (Ishikawa *et al.,* 2010; Ishikawa *et al.,* 2005). Ueno *et al.* (2009) also identified another major QTL for Cd accumulation in *O. sativa* that was mapped on the short arm of chromosome 7. QTLs for the translocation of Cd from roots to sink regions were reported in *O. sativa* (Xu *et al.,* 2012; Tezuka *et al.,* 2010). Tezuka *et al.* (2010) revealed a major QTL (*qCdT7*), mapped to chromosome 7, which controlled the translocation of Cd from roots to shoots. This QTL explained 88% of the phenotypic variation indicating that low Cd accumulation was a dominant trait. Dufey *et al.* (2009), using recombinant inbred lines, identified in *O. sativa* 24 putative QTLs involved in Fe tolerance which were mapped to chromosomes 1, 2, 3, 4, 7 and 11. In addition, two QTLs, located on chromosomes 2 and 3, were involved in As concentration in shoots and in roots respectively.

In durum wheat (*Triticum durum*, L.), Cd accumulation is controlled by a major gene named *Cdu1* and localized on chromosome 5BL (Knox *et al.,* 2009; Clarke *et al.,* 1997). Further, Ci *et al.* (2012) characterized 26 QTLs involved in Cd tolerance and accumulation in *T. aestivum*, where 16 were involved in Cd stress control, 8 for Cd tolerance and 2 for Cd accumulation in roots. In *A. sativa* L., a single QTL for Cd accumulation in grain has been reported (Tanhuanpää *et al.,* 2007).

In wheat (*T. aestivum* L.), Mayowa and Miller (1991) reported QTLs involved in Cu tolerance and accumulation that were mapped to chromosomes 5A, 4D, 7A, 7B, 7D. Ganeva *et al.* (2003) also characterized QTLs for *T. aestivum* on chromosomes 1A, 1D, 3A, 3B, 4A and 7D. Bálint *et al.* (2003) identified QTLs associated with Cu tolerance located on *T. aestivum* chromosomes 3D, 5A, 5B, 5D, 6B and 7D. In addition, Bálint *et al.* (2007) also determined QTLs for Cu tolerance in *T. aestivum*. They reported one major QTL for Cu tolerance on chromosome 5D and minor QTLs on chromosomes 1A, 2D, 4A, 5B and 7D. A QTL affecting shoot Cu content under Cu stress conditions was mapped on chromosome 1BL and an additional QTL for Cu accumulation was found on chromosome 5AL. The role of these genes located on various chromosomes in these different studies suggests that Cu tolerance is a polygenic character, as well as the possibility of different gene expressions against distinct toxic Cu concentrations in different populations. The accumulation of Cu in the shoots is affected by different QTLs, suggesting a strong metal-specific uptake and/or translocation. Bálint *et al.* (2007) reported a negative correlation between Cu tolerance and accumulation in the shoot indicating that the key tolerance mechanism in wheat could be the restriction of Cu uptake in the roots or the reduced translocation from root to shoot.

3.3. Categories of plants growing on metal contaminated soils

Baker and Walker (1990) categorized plants into three groups according to their strategy for coping with metal toxicity in soil; metal excluders, indicators and accumulators/ hyperaccumulators.

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3.3.1. Excluders

The metal excluder strategy consist in limiting the amount of metals translocated from roots to shoots thus maintaining low levels of metal concentration in their aerial parts. Large amounts of metals in the roots of excluder species have been reported (Baker and Walker 1990). Examples of excluder species include *Oenothera biennis, Commelina communis, Silene maritime*, *Agrostis stolonifera* L., woody plants such as *Salix*, *Populus* and *Pinus radiata* (Maestri *et al.,* 2010; Wei *et al.,* 2005).

3.3.2. Accumulators/hyperaccumulators

Metal accumulators/hyperaccumulators are plants that can concentrate metals in their above-ground tissues to levels that exceed those in the soil or also to those in the non accumulating species found growing nearby with concentrations up to 100 times more than non hyperaccumulators (Salt *et al.,* 1998). Accumulators/hyperaccumulators growing on metal contaminated environments can naturally accumulate higher levels of heavy metals in their shoots than in their roots (Kachout *et al.,* 2009). Some plants can accumulate only a specific metal while others can accumulate multiple metals ((Mganga *et al.,* 2011; Almås *et al.,* 2009). Presently, at least 45 plant families comprising more than 400 species have been found to accumulate metals in their harvestable tissues, and the majority of them belong to the *Brassicaceae* family (Pal and Rai 2010). The best known genera from this family are *Alyssum* and *Thlaspi*. *Thlaspi* species can accumulate more than 3% of their shoots in Zn, 0.5% in Pb and 0.1% in Cd. *A. halleri* can also accumulate more than 1% of its above-ground biomass in Cd and Zn and *Alyssum* species can accumulate over 1% Ni in their harvestable parts (Di Baccio *et al.,* 2011). There are variations among family, species and populations in the ability to accumulate metals. For example, *Arabidipsis halleri* can accumulate Cd and Zn in their harvestable parts where as *A. thaliana* is known to be a metal excluder and restricts metals in the roots. *Betula spp.* can accumulate Zn, while other trees species of the same family (*Carpinus* and *Corylus*) are unable to do so (Ernst, 2006; Ernst, 2004).

3.3.3. Indicators

Like accumulators, metal indicators accumulate metals in their aerial tissue, but the metal levels in the above ground tissue of these plants usually reflect the metal concentration in the surrounding environment (Baker and Walker 1990). If these plants continue to uptake metals, they will eventually die-off. These plants are of biological and ecological importance since they are pollution indicators and also, like accumulators, they absorb pollutants (Mganga *et al.,* 2011).

3.3.4. Determination of excluders, indicators and hyperaccumulators plants

A plant is classified as a hyperaccumulator when it meets four criteria including; a) when the level of heavy metal in the shoot divided by level of heavy metal in the root is greater than 1 (shoot/root quotient > 1); b) when the level of heavy metal in the shoot divided by

total level of heavy metal in the soil is greater than 1 (extraction coefficient > 1) (Rotkittikhun *et al.,* 2006; Harrison and Chirgawi 1989); c) when the plant takes up between 10 – 500 times more heavy metals than normal plants (uncontaminated plants - control plants) (Fifield and Haines 2000; Allen,1989); and d) more than 100mg/kg of cadmium, 1000g/kg of copper, lead, nickel, chromium; or more than 10000mg/kg of zinc (Mganga *et al.,* 2011; Ernst, 2006; Brooks, 1998). An excluder is a plant that has high levels of heavy metals in the roots but with shoot/root quotients less than 1 (Boularbah *et al.,* 2006). Finally, Baker and Walker (1990) classified a plant as an indicator when the levels of heavy metals within their tissues reflect those in the surrounding soil.

3.4. Physiological mechanisms of metal resistance

Resistant plants are able to grow on metal contaminated soil due to avoidance and/or tolerance strategies. Plant resistance to high levels of heavy metals in soils can result from either reduced uptake or once taken up, metals have to be transformed into a physiologically tolerable form.

3.4.1. Restriction of metal uptake

The plasma membrane is the first structure of living cells exposed to heavy metals. The membrane functions as a barrier for the movement of heavy metals into cytoplasm. The restriction of metals at the plasma membrane limits the uptake and accumulation of metals by preventing their entry into the cytoplasm. This can be done by changing the ion binding capacity of the cell wall and/or decreasing the uptake of metal ions through modified ion channels, and/or by removing metals from cells with active efflux pumps and/or with root with root exudates (Tong *et al.,* 2004).

3.4.1.1. The cell wall

The cell wall and membrane interface could be a site of metal tolerance since a significant amount of metals has been reported to be accumulated there. Divalent and trivalent metal cations can bind plant cell walls because of the presence of functional groups such as –COOH, -OH and –SH. Pectins are polymers that contain carboxyl groups which enable the binding of divalent and trivalent heavy metals ions. In enriched heavy metal environments, some plants will increase the capacity of their cell wall to bind metals by increasing polysaccharides, such as pectins (Colzi *et al.,* 2011; Pelloux *et al.,* 2007). Konno *et al.* (2010; 2005) showed that the pectin in root cell walls was important in binding Cu in the fern, *Lygodium japonicum,* and in the moss, *Scopelophila cataractae*. The cell wall of *Minuartia verna sp. hercynica* growing on heavy metal contaminated medieval mine dumps has been found to have high concentrations of Fe, Cu, Zn and Pb (Solanki and Dhankhar 2011; Neumann *et al.,* 1997). On the other hand, Colzi *et al.* (2012) found that a copper tolerant *Silene paradoxa* population restricted the accumulation of Cu in roots, when exposed to high Cu, by decreasing their pectin concentration in the cell wall and increasing pectin methylation thus preventing the binding of Cu.

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3.4.1.2. Root exudates

Resistant plants can also restrict the entry of metals by immobilizing them in the rhizosphere with root exudates outside the plasma membrane (Colzi *et al.,* 2011). This has been reported in *T. aestivum* where the exudation of phytochelatins, citrate and malate may be responsible for Cu exclusion mechanisms in non accumulators (Yang *et al.,* 2005b; Bálint *et al.,* 2007). Hall (2002) also proposed a mechanism for Ni exclusion in plants involving Ni-chelating exudates which include histidine and citrate. In non hyperaccumulator plants, these Ni chelators accumulate in their root exudates which, in turn decreases Ni uptake. The copper exclusion could be due to its chelation with citrate and malate exudates in the rhizosphere of wheat roots. The restriction of Cu uptake in wheat by the efflux of these organic acids has been previously documented by Nian *et al.* (2002).

3.4.2. Chelation

The phytotoxic effect of free metal ions can be eliminated by their chelation by specific high-affinity ligands (Yong and Ma 2002). The chelation of metals allows for the restriction of metal uptake, the uptake of metal ions, sequestration and compartmentation, as well as xylem loading and transport within the plant. Baker *et al.* (2000) categorized these ligands according to the characteristic electron donor centers, which include sulfur donor ligands, oxygen donor ligands and nitrogen donor ligands.

3.4.2.1. Oxygen donor ligands

Organic acids such as malate, aconitate, malonate, oxalate, tartrate and citrate are involved in metal uptake restriction and dexotification in plants. These carboxylic acid anions form complexes with divalent and trivalent metal ions with high stability. They are involved in the restriction of metal entry into the cell, metal exclusion in the root cells, accumulation and transport within the plants. In wheat (*T. aestivum*), citrate and malate formed complexes with Cu in order to immobilize this metal in the rhizosphere thus preventing its entry into the cell (Yong and Ma 2002). Citrate was also involved in the hyperaccumulation of Ni in 17 New Caledonian plants and the amount of citrate produced was highly correlated with the accumulation of Ni (Lee *et al.,* 1977). The accumulation of Zn in some plants is facilitated by the transport of malate-Zn complexes. Upon the Zn ions uptake into the cytoplasm, they are bound to malate, which serves as a carrier to transport the Zn ions to the vacuole. Once there, the Zn ions are complexed by a terminal acceptor and released from malate. The malate is then able to return to the cytoplasm and transport additional Zn ions to the vacuole (Yong and Ma 2002). Still and Williams (1980) reported that the transport of free Ni ions to root cells via membrane is restricted. However, when Ni is bound to organic compounds such as citric and malic acids, it can be transported across the plasma membrane (Yong and Ma 2002). In *Zea mays*, the production of organic acid is influenced by external aluminium ion concentration (Pintro *et al.,* 1997). Also, in manganese tolerant *T. aestivum* cultivars, the production of malic, citric or aconitic acid was not induced when exposed to this metal but for the manganese sensitive cultivars, the organic acids concentration slightly increased (Burke *et al.,* 1990).

3.4.2.2. Nitrogen donor ligands

This group consists of amino acids and their derivatives which have relatively high affinity for specific metals. Krämer *et al.,* (1996) revealed histidine to be involved in the Ni tolerance and translocation of the hyperaccumulator plant *Alyssum lesbiacum.* The majority of Zn in roots of the Zn hyperaccumulator, *T. caerulescens*, was complexed with histidine (Salt *et al.,* 1999). Studies have also shown histidine to be involved in the restriction of metal uptake. For example, plants chelate Ni with histidine in the rhizosphere which prevents the uptake of this metal (Wenzel *et al.,* 2003).

3.4.2.3. Sulfur donor ligands

In plants, sulfur donor ligands are composed of two classes of metal chelating ligands which are phytochelatins (PCs) and metallothioneins (MTs). Phytochelatins are small metal binding peptides synthesized from the tripeptide glutathione (γ-Glu-Cys)$_{2\text{-}11}$-Gly) (Solanki and Dhankhar 2011; Hall, 2002). Since there is a γ-carboxamide linkage between glutamate and cysteine, PCs are not synthesized by translation of mRNA, but rather it is a product of an enzymatic reaction involving the enzyme PC synthase (Yong and Ma 2002). The production of PCs is positively correlated with metal accumulation in plant tissues (Pal and Rai 2010). PCs are produced in cells immediately after heavy metal exposure, including Cd, Pb, Zn, Ag, Hg, As and Cu as seen in *Rubia tinctorum* (Maitani *et al.,* 1996). PC production can be induced in roots, shoots, and leaves as observed in *Sedum alfredii* when exposed to Cd (Pal and Rai 2010).

Several research groups concurrently and independently cloned and characterized genes encoding PC synthase. These genes were isolated from *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *T. aestivum*, and were designated *AtPSC1, SpPCS,* and *TaPCS1*, respectively. They encoded 50-55kDa sequences with 40-50% similarity. The polypeptides were found to be active in the synthesis of PCs from glutathione (GSH) (Yong and Ma 2002). In cultured *Silene cucubalis* cells, the presence of heavy metals, such as Cd, Cu, Zn, Ag, Hg and Pb, induce the synthesis of PCs by PC synthase from the GSH like substance (Pal and Rai 2010). Gaudet *et al.* (2011) did a comparative analysis of two *Populus nigra* genotypes from contrasting environments. They determined that both genotypes responded differently to Cd stress. The southern genotype (Poli) was more tolerant than the northern genotype (58-861). This variation was due to different adaptation strategies to Cd stress. The thiol and PC content, which was associated with the *glutathione S-transferase* gene, was higher in the southern genotype as compared to the northern genotype, which under Cd stress, revealed differences in the use of phytochelatin pathway that might be related to the variation in their Cd tolerance.

The second class of sulfur donor ligands are metallotioneins (MTs). They are low molecular weight (4-14kDa), cysteine-rich, metal-binding proteins found in a wide range of organisms (animals, plants, eukaryotic microorganisms, and prokaryotes) (Huang and Wang 2010). Unlike PCs, they are encoded by structural genes (Yong and Ma 2002). They play essential roles in a variety of organisms including Cu, Cd and Hg detoxification by sequestration (Palmiter, 1998; Ecker *et al.,* 1989), Zn homeostasis (Coyle *et al.,* 2002) and also scavenging of reactive oxygen species (Wong *et al.,* 2004). MTs have been divided into two classes based on their cysteine residue arrangements. Class I MTs are widespread in vertebrates and are

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composed of 20 highly conserved cysteine residues based on mammalian MTs. Class II MTs have slightly flexible cysteine arrangements and are found in plants, fungi and invertebrates. A third class includes phytochelatins (Chaturvedi *et al.,* 2012). Based on the position and allocation of cysteine residues, class II plant MTs are additionally divided into four types (Cobbett and Goldsbrough 2002). Type 1 plant MT genes have been more highly expressed in roots compared to leaves while the reverse is observed for the expression of type 2 plant MT genes. Type 3 MT genes are highly expressed in ripening fruits or in leaves while the expression of type 4 plant MT gene is restricted to developing seeds (Sekhar *et al.,* 2011; Cobbett and Goldsbrough 2002).

The expression of MT genes in plants subjected to metal stress has been studied. *AtMT1* and *AtMT2* genes showed increased expression levels when *Arabidopsis* plants were exposed to high levels of Cu and Cd (Sekhar *et al.,* 2011). Van Hoof *et al.* (2001) reported that the copper tolerant *S. vulgaris* individuals showed higher *SvMT2b* expression in roots and shoots when exposed to high concentrations of copper compared to the copper sensitive plants. Huang and Wang (2009) reported an increase in BgMT2 mRNA expression in large-leafed mangrove plants (*Bruguiera gymnorrhiza*) when exposed to Zn, Cu or Pb. Similar results were described by Gonzalez-Mendoza *et al.* (2007) in black mangrove *(Avicennia germinans*) seedlings exposed to Cd or Cu, showing a significant increase in AvMT2. High levels of the *CcMT1* transcripts were also observed in pigeon pea (*Cajanus cajan* L.) exposed to Cd and Cu (Sekhar *et al.,* 2011).

In general, there are variations between species in the expression of MTs to various metals. The up and down regulation of MTs in response to metal stress is largely unknown in plants. The MT gene expression was shown to be strongly induced by Cu, Cd, Pb and Zn (Huang and Wang 2009; Gonzalez-Mendoza *et al.,* 2007; van Hoof *et al.,* 2001). MT gene expression is also influenced by other abiotic stressors including absciscic acid (ABA), drought, salinity, heat, cold light, wounding and senescence (Sekhar *et al.,* 2011).

3.4.3. Mechanisms involved in internal metal tolerance

3.4.3.1. Metal uptake

The uptake of metal from soil into roots is dependent on the bioavailabilty of the metal, as well as its mobility in the rhizosphere (Maestri *et al.,* 2010). The bioavailability of various metals greatly varies. No correlation exists between the metal content in soils and in plants (Clemens, 2006). The bioavailability of metals in the rhizosphere is affected by the chemical environment. For example, in *T. caerulescens,* the chemical form of nitrogen influences the plants ability to uptake Cd and Zn (Maestri *et al.,* 2010; Xie *et al.,* 2009). Metals present in the rhizosphere of hyperaccumulators are more bioavailable than for those of non hyperaccumulators. Plants can render metals mobile in their rhizosphere by excreting root exudates, such as organic acids and phytosiderophores and by acidification with protons (Maestri *et al.,* 2010; Marschner, 1995). Bacteria in the soil also affect metal mobility and availability by lowering the pH, producing hormones, organic acids, antifungals, antibiotics and metal chelators which all enhance the root growth (Maestri *et al.,* 2010; Wenzel *et al.,* 2003). Higher amounts of bacteria were found in the rhizosphere of hyperaccumulators. Microorganisms found in the rhizo-

sphere were linked to an increased uptake of Cd, Z, and Pb in *Sedum alfredii* and an enhanced root growth (Maestri *et al.,* 2010; Xiong *et al.,* 2008).

3.4.3.2. Metal uptake across the plasma membrane

The uptake of heavy metals in plants is mediated by a group of metal transporter families which consists of iron-responsive transport proteins (ZIP-IRT), the heavy metal-transporting P_{1B}-type subfamily of P-type ATPases, the natural resistance associated macrophage proteins (NRAMP) and the cation diffusion facilitators (CDF) (Baxter *et al.,* 2003). Transporters were originally identified for Fe^{2+} or Zn^{2+} homeostasis, but it was demonstrated that most transporters of essential metal ions can also carry non essential metals, such as Cd (Zhou *et al.,* 2012). The uptake of non essential metals may be the result of their close chemical characteristics or metal ion size to essential metals. Some metal transporters, present in the plasma membrane of root cells, exhibit low substrate specificity which can lead to the accumulation of other metals in plants (Schaaf *et al.,* 2006). For example, the non-functional metal Cd can be taken up via a Ca^{2+} transporter (Perfus-Barbeoch *et al.,* 2002) or also via the Fe^{2+} transporter IRT1 (Korshunova *et al.,* 1999). Plant tolerance to metal stress can be achieved with the modification of these transporter activities (Zhou *et al.,* 2012). Plants can prevent the uptake of certain metals by down-regulating the expression of such transporters, as observed in *S. vulgaris,* where the tolerant plants restrict the uptake of Cu by the down-regulation of Cu-transporters (Assunçãno *et al.,* 2003; Harmens *et al.,* 1993). Since Fe and Ni belong to the group of transient metals and have similar chemical properties, Fe deficiency may be the result of Ni phytotoxicity. Ni competes with Fe in physiological and biochemical processes, and in turn roots, can uptake Ni by Fe transporters (Pandey and Sharma 2002).

Increased Zn uptake is driven by an overexpression of members of the ZIP family of transporters. Under Zn deficiency conditions, many members of the ZIP transporter family are overexpressed in non hyperaccumulator species, while in hyperaccumulators, they are independently expressed regardless of Zn supply (Verbruggen *et al.,* 2009). Nishida *et al.* (2011) and Schaaf *et al.* (2006) showed that *A. thaliana* can increase the uptake of Ni in roots when Fe levels are low by the Iron-Regulated Transporter 1 (AtIRT1; member of Zrt/IRT-like *ZIP* family of transporters). AtIRT1 has a wide specificity for divalent heavy metals including Ni, Zn, Mg, Co and Cd and mediates the accumulation of such metals under Fe-deficient conditions. Nakanishi *et al.* (2006) reported that Cd was uptaken in yeast by two *O. sativa* Fe^{2+} transporters, *OsIRT1* (Iron-Regulated Transporter 1) and *OsIRT2.*

The uptake of Ni of some Ni hyperaccumulator accessions of *Thlaspi goesingense*, *Thlaspi japonicum* and *T. caerulescens* has been reported to be inhibited in the presence of Zn. This demonstrated that Ni entered the cell via Zn uptake transporters, specifically the TcZNT1 transporter (Assunçãno *et al.,* 2008). In Zn deficiency conditions, the expression of AtZIP4, the orthologue of TcZNT1 in *A. thaliana,* can be induced but when additional Ni was added, the expression was repressed. This suggested that Zn and Ni competed for their uptake via AtZIP4/TcZNT1 transporters (Hassan and Aarts 2011). In addition, in presence of high Zn concentration, the expression of *ZNT1* was higher in Zn hyperaccumulator *T. caerulescens* roots than in the non hyperaccumulator *Thlaspi arvense* suggesting its involvement in the hyperac-

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cumulator phenotype (Hassinen *et al.,* 2007; Assunçãno *et al.,* 2001; Assunçãno *et al.,* 2001; Pence *et al.,* 2000). Milner *et al.* (2012) also determined that NcZNT1, isolated from *T. caerulescens*, played a role in Zn uptake from the soil which was based on its high expression in root.

Heavy metal-transporting P_{1B}-type transporters are also involved in metal-ion homeostasis and tolerance in plants by transporting essential and non essential heavy metals such as Cu, Zn, Cd, Pb across cell membrane. Transporters located at the plasma membrane function as efflux pumps by removing toxic metals form cytoplasm. They have also been found in membranes of intracellular organelles for compartmentalization of metals for sequestration in vacuoles, golgi or endoplasmic reticulum (Yang *et al.,* 2005b). These ion pumps transport ions across a membrane by hydrolysing ATP (Benitez *et al.,* 2012). Eight P_{1B}-ATPases, AtHMA1–AtHMA8, have been reported in *Arabidopsis* (Baxter *et al.,* 2003). AtHMA1, 2, 3, and 4 showed high similarity with $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ATPases previously characterized in prokaryotes (Axelsen and Palmgren 2001). The AtHMA4 was located at the plasma membrane. The ectopic expression of AtHMA4 improved the growth of roots in the presence of toxic Zn, Cd and Co concentrations (Yang *et al.,* 2005b). The heterologous expression of AtHMA4 enhanced Cd tolerance in yeast (Mills *et al.,* 2003).

In addition, the gene *Nramp* encodes for another divalent metal transporter located at the plasma membrane. This transporter also removes toxic metals from the cytosol by efflux pumping. It has been reported to be expressed in roots of *Arabidopsis* and *O. sativa* (*OsNramp1*- expressed in rice roots where as *OsNramp2* is expressed in leaves and *OsNramp3* is expressed in both tissues). The *OsNramp1* gene was found to be involved in the uptake of Mn, while the *Nramp* genes in *Arabidopsis* and rice were involved in the uptake of Cd, and other divalent metals (Yang *et al.,* 2005b). The AtNRAMP1, 3, and 4 showed uptake of Cd^{2+} when they were expressed in the yeast *Saccharomyces cerevisiae*. In addition, Cd^{2+} hypersensitivity was observed in *A. thaliana* when AtNRAMP3 was overexpressed. This transporter was located in the vacuolar membrane where it is involved in the mobilization of metals from the vacuole (Clemens, 2006).

In bacteria and in some eukaryotes, Zn, Co and Cd are transported by the CDF transport proteins. Within the *Arabidopsis* genome, there are 12 nucleotide sequences that are predicted to encode members of CDF transporter family. However, these transporters might be involved in cation efflux out of the cytoplasm, by pumping ions out of the cytoplasm to the exterior of the cell or into intracellular compartments such as the vacuole (Yang *et al.,* 2005b).

Plants can make metal ions more available for uptake by acidifying the rhizosphere and pumping protons via plasma membrane-localized proton pumps; and also by exuding low molecular weight (LMW) compounds that act as metal chelators (Clemens, 2006). The secretion of organic acids can render heavy metals mobile and enhance their absorption by plant roots. Krishnamurti *et al.* (1997) reported that when Cd was complexed with organic acids, it was readily available for transport across the membrane, while free Cd ions were restricted for uptake. Cieśliński *et al.* (1998) revealed a higher acetic acid and succinate in the rhizosphere of the *T aestivum* (Kyle) Cd accumulating genotype compared to the non accumulating (Arcola) wheat genotype. The Zn/Cd hyperaccumulating *Sedum alfredii* was able to extract high levels of Zn and Pb from its contaminated environment because of the release of root exudates (Li

et al., 2005). In *Alyssum*, the Ni transport and accumulation was enhanced by secretion of histidine in the rhizosphere (Krämer *et al.,* 1996).

3.4.3.3. Sequestration/compartmentation

Some metal tolerant plants can accumulate large amounts of metals within the cell without exhibiting toxicity symptoms (Entry *et al.,* 1999). These plants are able to store the surplus of accumulated metals where no sensitive metabolic activities occur such as organs or subcellular compartments (Ernst, 2006). This avoidance of metal poisoning involves the intracellular sequestration and apoplastic or vacuolar compartmentation of the toxic metal ions (Liu *et al.,* 2007). Compartmentation of metals can also be found in the cells central vacuole. This was observed in the Zn resistant *Deschampsia cespitosa* where the excess Zn ion was removed from the cytoplasm and actively pumped into the vacuoles of root cells where as Zn sensitive plants had a much lower capacity to do so (Brookes *et al.,* 1981).

Schaaf *et al.* (2006) determined that the transporter AtIREG2, located at the tonoplast, was involved in Ni detoxification in roots. AtREG2, confined to roots, prevents heavy metal translocation to shoots restricting metals to roots. This transporter counterbalances the low substrate specificity of transporter AtIRT1 and other iron transporters in iron deficient root cells. The AtIREG2 transporter, found in *A. thaliana*, was involved in the detoxification of Ni in roots under Fe deficiency conditions at pH 5 (Schaaf *et al.,* 2006). The *T. caerulescens ZTP1* gene was involved in the intracellular sequestration of Zn. The expression of the *ZTP1* gene was higher in the roots and shoots of the Zn tolerant *T. caerulescens* compared to the non tolerant plant (Assunçãno *et al.,* 2001).

Members of the CDF protein play a role in tolerance to various metals including Cd, Co, Mn, Ni and Zn by their sequestration into vacuoles (Montanini *et al.,* 2007). Increased Zn tolerance and accumulation was reported in non accumulator *A. thaliana* when *AtMTP1*, *PtdMTP1*, *AtMTP3* and *TgMTP1* (members of the CDF family) were ectopically or heterologously expressed. This suggested that the function of these proteins was the creation of a sink of Zn in the vacuole of plant cells in instances of high intracellular Zn levels or as buffer in Zn deficiency situations (Hassan and Aarts 2011).

Phytochelatins are also thought to be involved in the restriction of metals to the roots (Zenk, 1996). When *Nicotiana tubacum* seedlings were exposed to excess Cd, the level of phytochelatin increased (Vogelilange and Wagner 1990). The metal-phytochelatin complexes are formed when plants are exposed to high heavy metal concentrations. They are then sequestered into vacuoles for detoxification. A group of organic solute transporters actively transport phytochelatin-metal complexes into the plant's vacuole (Solanki and Dhankhar 2011; Salt and Rauser 1995). In the presence of excess Cu and Cd, phytochelatins form complexes with these metals in *Zea mays* and in turn reduce the root to shoot translocation (Galli *et al.,* 1996). The synthesis of phytochelatins is catalyzed by the enzyme phytochelatin synthase (PCS), a constitutive enzyme which requires post-translational activation by heavy metals and/or metalloids that include Cd, Ag, Pb, Cu, Hg, Zn, Sn, As and Au (Solanki and Dhankhar 2011). Martínez *et al.* (2006) reported that the expression of a PCS gene isolated from *T. aestivum* improved the

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accumulation of Cd, Pb and Cu in *Nicotiana glauca.* The elevation of phytochelatin concentration in roots might reduce the root to shoot transport required for accumulation in shoots.

3.4.3.4. Root to shoot translocation

The translocation of metals to the aerial biomass can be an important biochemical process used by plants to remediate polluted areas. In some plants, the mobilization of metals from their roots to their above aerial organs can minimize the damage that could be exerted by these heavy metals on the root physiology and biochemistry (Zacchini *et al.,* 2009). Excluders prevent or limit the translocation of toxic metals or essential metals from roots to shoots. On the other hand, accumulators/hyperaccumulators translocate metals from roots to shoots via the xylem with the transpiration stream. This is accomplished by increasing the uptake of metals in roots, and by reducing the sequestration of metals in the root.

The chelation of metals with ligands, such as organic acids, amino acids and thiols facilitates the movements of heavy metals from roots to shoots (Zacchini *et al.,* 2009). The xylem cell wall has a high cation exchange capability, thus the movement of metal cations is severely retarded when the metals are not chelated by ligands. Organic acids are involved in the translocation of Cd in the species *Brassica juncea* (Salt *et al.,* 1995).

The chelation of Ni to histidine is involved in the long distance translocation of Ni in the hyperaccumulator *A. lesbiacum*, where a 36-fold increase was reported in the histidine content of the xylem sap upon exposure to nickel (Solanki and Dhankhar 2011; Krämer *et al.,* 1996). Richau *et al.* (2009) found that the Ni hyperaccumulator, *T. caerulescens*, had a higher free histidine concentration in roots compared to the non Ni hyperaccumulator *T. arvense.* Also, *T. caerulescens* had less Ni in root vacuoles than *T. arvense* because the histidine-Ni complexes were much less taken up by vacuoles than free Ni ions. Therefore, an increase in free histidine in roots inhibited the vacuolar sequestration of His-Ni in *T. caerulescens* compared to free Ni in *T. arvense* and also had enhanced histidine-mediated Ni xylem loading. The elevated free histidine in root cells appears to be involved in reduced vacuolar sequestration and enhanced xylem loading of Ni (Richau and Schat 2009). This was also the case for Zn and Cd for this hyperaccumulating species (Hassan and Aarts 2011). An increase in Ni accumulation was also observed in the Ni hyperaccumulator *Sebertia acuminata* where, when chelated to citrate, Ni was able to translocate to the shoot. In the absence of citrate, Ni was no longer accumulated in the aerial tissues (Lee *et al.,* 1977).

The chelation of metals with nicotianamine (NA) also contributes to improved tolerance. Nicotianamine can chelate and transport divalent Ni, Cu and Zn (Takahashi *et al.,* 2003; Pich *et al.,* 2001; Ling *et al.,* 1999). The nicotianamine synthase (NAS) enzyme is responsible for the synthesis of NA by trimerization of S-adenosylmethionine (Shojima *et al.,* 1990). When exposed to high levels of Zn, Cd, and/or Ni, all four *NAS* genes were highly expressed in *T. caerulescens* compared to non hyperaccumulator *A. thaliana* (van de Mortel *et al.,* 2006). In the presence of elevated Mn, Zn, Fe and Cu concentrations, Kim *et al.* (2005) reported an increased expression of the *NAS* gene, as well as NA levels for *A. thaliana* and *N. tubacum*. In addition, Pianelli *et al.* (2005) showed that the over-expression of the *T. caerulescens NAS3* gene in the Ni excluder *A. thaliana* resulted in improved Ni tolerance and Ni accumulation in their aerial organs. An

increase of Fe, Zn and Cu accumulation in *O. sativa* was associated with an overexpression of the *NAS3* gene (Hassan and Aarts 2011; Kawachi *et al.,* 2009).

Visioli *et al.* (2010) also showed that metallothioneins may be involved in the translocation of Ni in *T. caerulescens.* An increase in MT-1B in the individuals from the metal contaminated environment was observed when metallicolous *T. caerulescens* and non-metallicolous *T. caerulescens* individuals were grown in presence of high Ni concentrations, compared to non contaminated site. Additionally, Visioli *et al.* (2012) analyzed four *T. caerulescens* sub-population (MP1 to MP4) for their ability to accumulate and tolerate Ni. In four sub-populations analyzed, MP2p translocated the highest amount of Ni to the shoots. This sub-population also had the highest level of putative metallothionein protein (MT4C). Constitutively higher expressions of other MTs are also seen in the hyperaccumulators *A. halleri*, *S. paradoxa* and *S. vulgaris*.

Transporters are not only involved in the uptake of metals from the soil, but also in their transport out of the vacuole. These mobilized metals can then be translocated to aerial tissue. Visioli *et al*. (2012) subsequently found for sub-population MP2p, which exhibited the highest level of Ni translocation of the four sub-populations analyzed, significantly higher levels of the ABC27 transporter. This transporter is part of the ABC family of transporters which are involved in removing metals from the cytoplasm by pumping outside the cell wall, metals sequestered in vacuoles and other subcellular compartments (Visioli *et al.,* 2012; Martinoia *et al.,* 2002; Sanchez-Fernandez *et al.,* 2001). Hassinen *et al.* (2007) showed that the AtMRP10 homolog, also part of the ABC family of transporters, had different expression in roots of two *T. caerulescens* populations with contrasting Zn tolerance and accumulation. In addition, the AtNramp3 transporter was also involved in the mobilization of vacuolar Cd back into the cytosol. This was oberved when *AtNramp3* was overexpressed in *A. thaliana*. AtNramp3 was further hypothesized to play a role in the mobilization of Fe, Mn, and Zn in the vacuole (Clemens, 2006).

The passage of metal ions and/or metal ligand complexes from the cytosol of root cells into the vascular tissue requires their transport across the cell membrane. Transporters involved in this activity are the heavy metal transporting P-type ATPases (HMAs) (Clemens, 2006). The AtHMA2 and 4 are involved in translocation of Zn in *A. thaliana*. Stunted growth and chlorosis resulted in the *hma2hma4* double mutant from inadequate Zn supply to the leaves. The two genes were expressed in vascular tissue which indicates their hypothesized function in xylem loading (Hussain *et al.,* 2004). The AtHMA4 transporter was also involved in the transport of Cd^{2+} ions (Clemens, 2006). In *T. caerulescens*, the P-type ATPase, TcHMA4, was also involved in the translocation of Zn. When Zn and Cd levels were elevated or when Zn is deficient, the expression of *TcHMA4* was induced in the roots. This transporter was involved in the xylem loading of Zn in plant roots (Hassinen *et al.,* 2007; Papoyan and Kochian 2004). Milner *et al.* (2012) also determined that NcZNT1 in *T. caerulescens* was not only involved in Zn uptake from the soil but also could be involved in the long distance transport of Zn from root to shoot via the xylem.

3.4.3.5. Metal storage

Metals have to undergo a xylem unloading process prior to their distribution and their detoxification in the shoot and their redistribution via the phloem (Schmidke and Stephan

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1995). Once unloaded, the metals are either taken up into surrounding cells and are symplastically transported through the leaf tissues or they are apoplastically distributed over the leaf (Hassan and Aarts 2011; Marschner, 1995). NA is important in the chelation of metals for their symplastic transport through the leaf. This occurs through the Yellow Stripe Like proteins (YLS) (Hassan and Aarts 2011; DiDonato *et al.,* 2004). In the hyperaccumulator *T. caerulescens*, three *YSL* genes (*TcYLS3*, *TcYSL5* and *TcYSL7*) were highly expressed in shoots around vascular tissues. This high level of expression was not observed in the excluder plant *A. thaliana* orthologues (Hassan and Aarts 2011; Gendre *et al.,* 2007). For the *TcYSL3*, it was suggested that its function was to unload Ni-NA complexes from the xylem into leaf cells and to distribute it to storage cells. Using yeast complementation and uptake measurement studies, it was determined that TcYSL3 was also a Fe/Ni-NA influx transporter. Considering that YSL proteins have a role in the transport of Fe-NA complexes, it was proposed that they might also be involved in the hyperaccumulation of Fe-NA in some plants (Hassan and Aarts 2011; Curie *et al.,* 2009).

The sequestration of excess essential and non essential metals is localized in various parts of the aerial tissue, such as trichomes, leaf epidermal cell vacuole and mesophyll vacuole. Broadhurst *et al.* (2004) grew five *Alyssum* hyperaccumulator species/ecotypes on Ni-enriched soil and determined that the majority of hyperaccumulated Ni was stored in either leaf epidermal cell vacuoles or in the basal section of stellate trichomes. They also found that the metal concentration in the basal part of the trichome was 15% to 20% of dry weight. This was among the highest metal concentrations reported in healthy vascular plant tissues. In *A. halleri*, the majority of Zn ions were stored in the vacuoles of mesophyll cells, while for *T. caerulescens*, most Zn ions were located in the vacuoles of epidermal cells (Verbruggen *et al.,* 2009). The transport of metals through the phloem sap is less documented. The sole molecule identified as a phloem metal transporter is nicotianamine which is involved in the transport of Fe, Cu, Zn and Mn (Stephan *et al.,* 1994).

3.4.4. Antioxidative defence involved in metal tolerance

In environments, where metals are present in toxic levels, the elevated activities of antioxidant enzymes and non-enzymatic constituents are important in the plant tolerance to stress. Metal tolerance may be enhanced by the plant's antioxidant resistant mechanisms. There is an indication that the alleviation of oxidative damage and increased resistance to stresses in the environment is often correlated with an effective antioxidative system. The minimization of damage due to oxidative stress is a universal feature of plants defense responses (Kachout *et al.,* 2009). The detrimental effect of heavy metals in plants is due to the production of ROS and induction of oxidative stress. Oxidative stress is expressed by the increase levels of reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Salin, 1988). ROS are strong oxidizing agents that lead to oxidative damage to biomolecules, for instance lipids and proteins and can eventually result in cell death (Gunes *et al.,* 2006). It is shown that plant tolerance to metals is correlated with a rise in antioxidants and activity of radical scavenging enzymes (Kachout *et al.,* 2009). Plants respond to oxidative stress by activating antioxidative defence mechanisms which involve enzymatic and non-enzymatic antioxidants. The enzymatic components include superoxide

dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and enzymes of ascorbate glutathione cycle whereas the non-enzymatic antioxidants include ascorbate and glutathione and atocoperol (Solanki and Dhankhar 2011; Kachout *et al.,* 2009). These antioxidants are responsible for elimination and destruction of the reactive oxygen species (Solanki and Dhankhar 2011).

Oxidative damage could result when the balance between the detoxification of the ROS products and the antioxidative system is altered (Kachout *et al.,* 2009). The tolerance of deleterious environmental stresses, such as heavy metals, is correlated with the increased capacity to scavenge or detoxify activated oxygen species (Kachout *et al.,* 2009). Boominathan and Doran (2003a,b) determined the role of antioxidative metabolism of heavy metal tolerance in *T. caerulescens*. They determined that superior antioxidant defenses, mainly catalase activity, may have an important role in the hyperaccumulator phenotype of *T. caerulescens*. Kachout et al (2010) determined the effects of Cu, Ni, Pb and Zn on the antioxidative defense systems of *Atriplex* plants. They found that when the plants were exposed to different levels of metals, their dry matter production and shoot height decreased. Of the antioxidant enzymes, metal toxicity only diminished the levels of superoxide dismutase (SOD) and probably ascorbate peroxidase (APX) but increased the activity of catalase (CAT) and glutathione reductase (GR). The plants showed an intermediate level of tolerance to the metal stress conditions imposed. The antioxidative activity may be of fundamental significance for the *Atriplex* plants in their response against environmental stress.

3.5. Problems associated with plant metal tolerance

Soils enriched with metals are demanding on tolerant and accumulator plants. The costs associated with their adaptation to these sites are related to energy and resources allocations. When a metal tolerant or accumulator plant is growing in a metal contaminated soil, there is an increase in cost because the organism has to spend energy to counter the effects of the metals (Maestri *et al.,* 2010). Slow growth and low reproduction are the main characteristics of plants growing on metal enriched soils (Ernst, 2006; Ernst *et al.,* 2000). Haldane (1954) stated that costs are associated with the natural selection of new alleles. More energy and resources are required for the maintenance of the tolerance mechanisms at the cellular level. It has been demonstrated that tolerant plants have increased synthesis of complexing molecules in the cytosol. For example, metallothioneins and phytochelatins for the detoxification of metals such as As, Cd, and Cu. ATP are also needed for the active transport of metals across the plasma membrane and tonoplast. The synthesis of these agents withdraws N, S and energy from the primary metabolism (Ernst, 2006; Verkleij *et al.,* 1998). Energy is also required for the translocation of metals from root to shoot as well as for their allocation to various tissues and cell types. The reduced biomass of metal tolerant plants compared to their non metal tolerant ancestors might also be the result of less than favourable environmental conditions such as low water and nutrient supply. The diminished biomass and seed production might be the result of all costs associated with their survival to these metal contaminated sites, such as adaptation and environmental constraints (Ernst, 2006). Plants have an advantages growing on metal contaminated soil. As previously mentioned, there is a lack of competitive species on these sites. With high metal accumulation of metals in their

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aerial tissues, the "elemental hypothesis" speculates that hyperaccumulators can deter predators such as herbivores from feeding on them (Maestri *et al.,* 2010; Vesk and Reichman 2009). However, some insects feed on hyperaccumulator plants and in turn accumulate the metals in their tissue which then aid in their defence against predators (Maestri *et al.,* 2010). This contradiction may explain why there is a mix of excluders, accumulator and hyperaccumulators growing on metal contaminated sites. Another advantage of hyperaccumulation is the elimination of competitive plants by further contaminating the surrounding soil by shedding their metal contaminated leaves (Maestri *et al.,* 2010).

3.6. Effects of metals on plant population diversity and structure

Elevated accumulations of metals in soil and vegetation have been documented within short distances of the smelters compared to control sites (Nkongolo *et al.,* 2008; Gratton *et al.,* 2000). Several authors have reported differences in genetic structure of plants growing in contaminated areas (Vandeligt *et al.,* 2011; Nkongolo *et al.,* 2008; Scholz and Bergmann 1984). Enzymatic studies of Norway spruce (*Picea abies*) revealed genetic differences between groups of sensitive trees in polluted areas (Scholz and Bergmann 1984). It has been demonstrated that the evolution of heavy metal tolerant ecotypes occurs at an unexpectedly rapid rate (Wu *et al.,* 1975) and that despite founder effect and selection, in several cases, the recently established tolerant populations maintain a high level of variation and appear to be at least as variable as non tolerant populations. Observations of higher heterozygosity in tolerant plants of European beech (*Fagus sylvatica*) in Germany (Muller and Starck 1985), scots pine (*Pinus* sylvestris) in Germany and Great Britain (Geburek *et al.,* 1987), trembling aspen (*Populus tremuloides*) and red maple (*Acer rubrum*) in the United States (Berrang *et al.,* 1986) have been reported. Several studies, however, have reported the detection of bottleneck effects (Nordal *et al.,* 1999; Vekemans and Lefebre 1997; Mejnartowicz, 1983). Mejnatowicz (1983) presented evidence of loss of genes and heterozygosity in tolerant Scots pines. The frequent lack of a bottleneck effect has been explained by different hypotheses: successive colonization events, a high number of tolerant plants in the primary populations, pollen flow from the neighboring populations, environmental heterogeneity and human disturbance (Nkongolo *et al.,* 2007).

Molecular analyses of several conifer and hardwood species clearly indicated that the exposure to metals for more than 30 years has no effect on genetic structure and diversity of early generations of *Picea mariana, P. glauca, Pinus banksiana, P. rubens, P. strobus,* and several hardwood populations in Northern Ontario (Narendrula *et al.,* 2012; Nkongolo *et al.,* 2012; Dobrzeniecka *et al.,* 2011; Vandeligt *et al.,* 2011). This lack of association between the level of genetic variation and metal content can be attributed to the long life span of these tree species. Table 1 shows similar level of genetic variabilities in pine populations growing in metal contaminated sites for more than 30 years compared to control in Northern Ontario, Canada. This is in contrast to data observed in herbaceous species such as *D. cespitosa* where a high level of metal accumulation reduced significantly the level of genetic variation (Table 2) (Nkongolo *et al.,* 2007). Metals impose severe stress on plants, especially in the rooting zone, which has led to the evolution of metal resistant ecotypes in several herbaceous species like *D. cespitosa* (Cox and Hutchinson 1980).

Populations	P (%)	h	I	Ne	Na
Vale site 1 (metal contaminated)	31.25	0.1120	0.1653	1.2035	1.3125
Vale site 2 (metal contaminated	31.25	0.1171	0.1727	1.2061	1.3125
Xtrata 2 (metal contaminated)	27.08	0.0995	0.1467	1.1758	1.2708
Xtrata 3 (metal contaminated)	20.83	0.0630	0.0982	1.1004	1.2083
Vale Tailing (metal contaminated)	35.42	0.0977	0.1552	1.1514	1.3542
Temagami site (control)	29.17	0.0818	0.1284	1.1310	1.2917
Low Water Lake (control)	31.25	0.0812	0.1297	1.1256	1.3125
Mean	31.63	0.1001	0.1528	1.1679	1.3163

Table 1. Genetic variability parameters of *Pinus banksiana* populations growing in the Sudbury, Ontario (Canada) area based on ISSR data.

P represents percentage of polymorphic loci; h, Nei's gene diversity; I, Shannon's information index; Ne, effective number of alleles; Na, observed number of alleles.

Region	Site	Polymorphism per site (%)	Mean polymorphism per region (%)
Sudbury	Coniston	72	74 (Sudbury)
(moderately contaminated)	Xtrata	92	
	Copper Cliff	67	
	Walden	65	
Cobalt	Cobalt-3	48	46 (Cobalt)
(highly contaminated)	Cobalt-4	46	
	Cobalt-5	44	
Manitoulin	Little Current	70	69 (Manitoulin)
(control)	Mississagi Lighthouse	68	

Table 2. Genetic variability within *Deschampsia cespitosa* populations from Northern Ontario generated with ISSR primers.

P represents percentage of polymorphic loci; Sudbury and Cobalt regions were moderately and highly contaminated with metals, respectively. Manitoulin Island region was not contaminated with metals and was used as a control region.

4. Conclusion

Plants play an essential role in the remediation of metal enriched soils. Coping mechanisms developed by some group of plants growing on metal contaminated soil facilitate the establishment of sustainable ecosystems in areas that would otherwise remain barren. A number

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of studies have been completed to explain the complex mechanisms involved in tolerance genotypes, and also the biological variability in their environmental adaptation. Depending on the circumstances, metal excluders or hyperaccumulators may be used to remediate polluted soil. Excluders may be useful for soil stabilization by preventing wind and water erosion and also by limiting the entry of heavy metals in the food chain. Metals can also be extracted by hyperaccumulators but since majority of these plants have low biomass, the extraction of metals from soil is very slow. The remediation of these sites using this technique may take up to hundreds of years. With genetic engineering, it may be possible to design the ideal plant prototype for the remediation of metal contamination in different environments. Many genes and mechanisms have been identified to have a role in tolerance and hyperaccumulation of metals. However, there is still a need for a better understanding of the mechanisms such as characterization of promoters of genes controlling metal tolerance and hyperaccumulation. This new knowledge would significantly contribute to a better understanding of the regulation and expression of different genes in hyperaccumulators. It is essential to mimic this regulation and expression of genes in high biomass non hyperaccumulators in order to obtain the hyperaccumulator phenotype.

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