Journal of Sciences, Islamic Republic of Iran 17(3): 203-214 (2006) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Uptake, Transport and Chelation of Cu and Zn at Toxic Levels in Tolerant and Sensitive Species from North West of Iran

R. Hajiboland,[1,*] V. Niknam,[2] H. Ebrahim-Zadeh,[2] and A. Mozafari[1]

[1] *Department of Plant Science, Faculty of Natural Sciences, Tabriz University, Tabriz, Islamic Republic of Iran*
[2] *Department of Biology, Faculty of Sciences, Tehran University, Tehran, Islamic Republic of Iran*

Abstract

From flora of North West of Iran, four contrasting species in term of frequency and coverage on heavy metal rich soils were selected to study physiological mechanisms for different tolerance of Zn and Cu toxicity in a hydroponic culture experiment. For study of Zn toxicity, *Dactylis glomerata* and *Secale monatum* and for Cu, *Cichorium intybus* and *Astragalus echinops* were selected. A clear difference was observed between *S. monatum* and *D. glomerata* for tolerance to Zn toxicity and between *C. intybus* and *A. echinops* for Cu toxicity. Higher sensitivity to Zn and Cu toxicity in *D. glomerata* and *C. intybus* was associated with higher Zn and Cu accumulation in root and shoot. In order to determine the possible role of chelating/detoxifying molecules, changes in the content of amino and organic acids in response to metal treatment were studied. A relative high citrate and malate content in *D. glomerata* in comparison with *S. monatum*, was not the reason of higher Zn tolerance. In contrast, endogenous malate content in *A. echinops* was accompanied by higher Cu tolerance in this species. Cysteine was the only possible chelating/detoxifying molecule for Cu, but not for Zn. Our results showed that a high Cu tolerance in *A. echinops* was associated with higher endogenous and inducible cysteine content of shoot. Activity of peroxidase decreased under Cu toxicity, interestingly, this reduction was much higher in *C. intybus* (susceptible) when compared with *A. echinops* (tolerant). Because of possible role of lignin in establishing an apoplasmic transport barrier, higher uptake and transport of Cu into shoot in *C. intybus* could be due to the result of stronger inhibition of guiacol peroxisdase *i.e.* inhibition of lignin formation, in this species under Cu toxicity.

Keywords: Heavy metals; Uptake; Transport; Chelation; Organic acids; Amino acids

Introduction

Environmental pollution by heavy metals occurs as a consequence of mining, manufacture and disposal of mineral ores, metal products and waste [4]. Heavy metals are present in the soils as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides, or they may constitute a part of the structure

[*] *E-mail: ehsan@tabrizu.ac.ir*

of silicate materials (indigenous soil content) [61].

Adaptation to high concentrations of heavy metals may be achieved in principle by exclusion of the elements from uptake in the roots or, after uptake, by specific compartmentation and detoxification [17]. Effects of elevated Zn concentrations on growth and physiological characteristics of leaves have been investigated [10,15,39,42,44,59,67]. Of particular importance for Zn tolerance is the subcellular localization of Zn [69].

The physiological causes of the phytotoxic effects of Cu have also been the subject of numerous studies [1,52-54,60]. The response of plants to Cu may depend on how the ions are distributed between shoot and root and between tissues within organs. A large proportion of the Cu absorbed by the plants is retained in the roots [26], therefore, while roots reflect Cu concentrations in the soil, Cu levels in the shoot do not show significant changes in response to change in soil Cu concentration [35].

Phytochelatins (PCs) are induced by a wide range of metal ions, the best activators are Cd followed by silver (Ag), bismuth (Bi), lead (Pb), zinc (Zn), copper (Cu), mercury (Hg) and gold (Au) cations [19]. However, accumulation of PCs is not necessarily associated with heavy metal tolerance. The mechanism of metal detoxification is more complex than simply chelation of metal ion by PCs. The metal ion must activate PC syntheses, be chelated by the PCs and then be transported to the vacuole and form a more complex aggregation in the vacuole. Although PCs appear to be induced by Cu and also chelate Cu *in vitro*, it is unknown whether PCs effectively chelate Cu *in vivo* or PCs-Cu complexes (if formed) can be sequestered in the vacuole. Mutants deficient in PCs, display only slightly elevated sensitivity to Cu [29]. On the other hand, induction of PCs synthesis results in consumption and therefore depletion of the glutathione pool [48], therefore leads to oxidative damage of the cells [24]. Such an effect was observed in Cu-sensitive populations of *Silene cucubalus* versus tolerant populations with lower PCs synthesis [24].Binding to PCs plays no role in Zn tolerance based on several studies and observations [32,39].

Due to the reactivity of metal ions with S, N and O, carboxylic and amino acids can act as potential ligands. Citrate, malate and oxalate have been implicated in a range of processes, including differential metal tolerance, metal transport through the xylem and vacuolar metal sequestration [62]. Citric acid, for instance, has been hypothesized to be a major Cd^{2+} ligand at low Cd^{2+} concentrations [70] and has been shown to form complexes with Ni^{2+} in Ni-hyperaccumulating plants [64]. In Zn-tolerant species, a Zn-malate shuttle has been proposed, in which Zn is more efficiently transported to the vacuole due to a higher concentration of malate in the cytosol [39,47,63]. However, the malate concentrations in roots of tolerant and sensitive *Silene vulgaris* were not consistently different and were not affected when plants were exposed to Zn [33]. In contrast, Godbold [27] demonstrated a high correlation between citrate content and Zn accumulation in *Deschamsia caespitosa* and argued that citrate may play a role in Zn tolerance. Moreover, Wang *et al.* [71] concluded that citrate has a high potential for complexing Zn, and that cytosolic citrate more likely play a role as a shuttle for transporting Zn into the vacuole. It appears that Zn is complexed mainly with histidine in the roots, whereas citrate is involved in binding about 20-40% of the total Zn in the xylem sap and leaves. Once Zn delivered to the shoots, it is preferentially sequestered in the vacuoles [39,40,68] and soluble Zn reaches a concentration of 385 mM in this compartment [40].

Evidence on the chelation of Cu with some low molecular weight organic compounds such as nicotianamine was obtained in nutritional range of supply [59]. However, for Cu at toxic levels, there is no direct evidence either for accumulation of organic and amino acids or any role in chelation or detoxification of this metal. with the exception of Al tolerance [23], unequivocal evidence for a function of organic acids in plant metal tolerance has been difficult to obtain, clear correlations between the concentration of a particular organic acid and the degree of exposure to a particular metal which have to be postulated in the light of metal specificity of most homeostatic and tolerance mechanisms, have not been observed [18].

Plant communities growing over Cu/Pb/Zn (base metal) deposits are called Zn-flora. In many cases it cannot be established whether or not the characterization flora is due to the presence of one or all of the three base metals mentioned above since all three are usually found together in areas of sulphide mineralization [9].

Hyperaccumulators are endemic to metalliferous soils, and metal concentrations in their aerial parts are one to three orders of magnitude greater than the non-accumulating species growing under the same environment [9]. Eighteen Zn hyperaccumulators have been reported and are defined as being able to accumulate more than 10,000 μg Zn g^{-1} in the above-ground parts on a dry weight basis in their natural habitat [2]. Zn hyperaccumulator plants are found in Zn/Pb rich soils, which are either developed for Zn/Pb mineral deposits or contaminated by industrial activities [72].

Flora of Cu rich soils has been described by some authors [6-8]. They have reported the presence of over 50 species endemic to Cu/Co deposits including several individual plants that have been used for geobotanical prospecting [9].

Our previous work on the flora of heavy-metal rich soils in North West Iran [30] showed that, there is a clear difference among some plant species in terms of coverage and frequency on metal-rich soils. While some species present with a high frequency and coverage on metal-rich soils and grow directly on mining areas, some other species are absent from these soils or restricted to marginal areas. From these two contrasting species *Secale monatum* and *Astragalus echinops* were from important elements of Zn and Cu -rich soils respectively, while *Dactylis glomerata* and *Cichorium intybus* were either absent or restricted to marginal areas of soils rich from these metals.

The investigation of Zn and Cu uptake, transport and chelation in four selected species, were the main objectives of this work.

Materials and Methods

Plant Materials

Two species were used in this work to study the Zn toxicity including *Secale monatum* and *Dactylis glomerata*. To study the Cu toxicity, *Astragalus echinops* and *Chicorium intybus* were selected. Seeds were collected from Zn rich areas near Khoy (West Azarbaijan Province, Iran) and Cu rich areas near Ahar (Mazraeh, East Azarbaijan Province, Iran).

Plant Culture

The experiments were conducted in a growth chamber with temperature programs of 25°/18°C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and at a light intensity of 300 μmol $m^{-2}s^{-1}$ (PPFD). Surface-sterilized seeds were germinated in the dark on vermiculite or filter paper, moistened with distilled water and 0.05 mM $CaSO_4$. The 7-12 days old seedlings with uniform size were transferred to hydroponic culture in plastic containers with 2L of nutrient solution (25%) and pre-cultured for 3 days. Metal treatments were started when plants were 10-15 days old. Five levels of $ZnSO_4$ or $CuSO_4$ at 0 (control), 25, 50, 75 and 100 μM were used. The composition of nutrient solutions for *Secale* and *Dactylis* was selected according to the nutrient solution applied for wheat [11] and contained (mM): $Ca(NO_3)_2$ 2.0, $MgSO_4$ 1.0, K_2SO_4 0.9, KH_2PO_4 0.25, KCl 0.1 and (μM): H_3BO_3 2.0, $MnSO_4$ 0.4, $ZnSO_4$ 1.0, $CuSO_4$ 0.4 and $(NH_4)_6Mo_7O_{24}$ 0.04 (pH=7.0). The composition of nutrient solutions for *Astragalus* plants was selected according to the composition applied for lupine [51] and contained (mM): $Ca(NO_3)_2$ 5.0, $MgSO_4$ 1.25, K_2SO_4 1.75, KH_2PO_4 0.25, KCl 0.25 and (μM): H_3BO_3 25, $MnSO_4$ 1.5, $ZnSO_4$ 1.5, $CuSO_4$ 0.5 and $(NH_4)_6Mo_7O_{24}$ 0.025 (pH=7). The composition of nutrient solution for *Chicorium* was selected according to the method applied for sunflower [21] and contaied (mM): $Ca(NO_3)_2$ 2.0, $MgSO_4$ 0.5, K_2SO_4 0.1, KH_2PO_4 0.1, KCl 0.1 and (μM): H_3BO_3 0.1, $MnSO_4$ 0.5, $ZnSO_4$ 0.5, $CuSO_4$ 0.2 and $(NH_4)_6Mo_7O_{24}$ 0.01 (pH=6.5). Iron was supplied as Fe(III)-EDTA (Ethylenediamine tetraacetic acid) at 100 μM (*Secale*, *Dactylis* and *Chicorium*) or 20 μM (*Astragalus*).

Nutrient solutions were changed every 4 days and pH of nutrient solutions were adjusted every day.

Harvest

Twelve days after treatment, plants were harvested. For removing of the Zn and Cu from root apoplasm, plants were placed for 1h in 5 mM $CaCl_2$+25% nutrient solution. Then, roots were washed with distilled water, plants were divided into shoots and roots, weighed and blotted dry on filter paper and dried at 70 °C for 2 days to determine dry weight. For determination of Zn and Cu content, oven-dried samples were ashed in a muffle furnace at 500°C for 8h and then digested in 1:3 (V/V) HNO_3. The digested samples were dried on a heating plate and subsequently ashed at 500°C for another 3h. Samples were resuspended in 2 ml 10% HCl and made up to volume by double-distilled water. Zn and Cu concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500).

Thin Layer Chromatography (TLC)

Fresh tissue samples were extracted in 20 mM Tris-HCl (pH=8.0) (5:1 buffer volume:fresh mass). The crude extract was centrifuged and applied to the plates based on the method of Oven *et al.* [55].

For the investigation of organic acids, 20 μl of tissue extract was applied to a cellulose plate (20×20 cm, Merck). The plate was placed in a glass chamber containing the mobile phase formic acid/ethanol/water (48.8:48.8:2.4, v/v/v). Following resolution, the plate was thoroughly dried for 1 day at room temperature and sprayed with a dichlorophenol-indophenol reagent (100 mg 2,6-dichlorophenol-indophenol in 100 ml ethanol). Blue spots on the plate indicate the presence of organic acids. The standard solutions of citric and malic acid

were applied simultaneously to the plates.

TLC for amino acids analysis was performed by resolving 20 µl of supernatant on a silica gel plate (20×20 cm, Merck). The plate was placed in a glass chamber containing the mobile phase chloroform/ methanol/25%ammonium hydroxide/water (40:40:10: 10, v/v/v/v). Another TLC experiment was conducted for a better resolution using the mobile phase isobuthanol/acetic acid/water (50:25:25, v/v/v). Following separation, the plate was air dried and sprayed with a ninhydrin reagent (200 mg nin-hydrin in 100 ml ethanol). Red spots indicate the presence of amino acids. The standard solutions of histidine, cysteine, glutamine and asparagines were applied simultaneously to the plates.

Quantification of organic acids and amino acids were estimated by modified densitometric thin-layer chromatography [45]. In this method, after visualizing of spots under UV chamber and determination of suitable wavelength, the plates were scanned by Shimadzu CS-9000 densitometer. The presence or absence as well as relative amount of each compound were reported using − or + characters. Each TLC experiment was conducted in triplicate.

Cysteine Assay

Cystein was extracted from fresh tissue in 20 mM Tris-HCl (pH=8.0) (5:1 v/w). Cysteine content was determined using a spectrophotometric method [28]. Sixty microliters of 50 mM DTT were added to 60 µl of tissue extracts. The sample was incubated at room temperature for 15 min, then 120 µl acetic acid and 120 µl acid-ninhydrin reagent (250 mg ninhydrin with 6 ml acetic acid and 4 ml HCl) were added to the sample. The assay mixture was incubated at 95°C for 10 min, after which cold ethanol (840 µl) was added. The mixture was then chilled on ice and the absorbance was determined at 560 nm.

Assay of Enzymes Activity

Activity of enzymes was assayed only in Cu treated plants.

Catalase: Catalase (EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease of absorbance at 240 nm [46,66]. The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The assay solution contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the change in absorbance was followed for 2 min. Unit activity was taken as the amount of enzyme which decomposes 1 M of H_2O_2 per min.

Peroxidase: Peroxidase (EC 1.11.1.7) activity was determined using the guaiacol test [12]. The tetraguaiacol formed in the reaction has an absorption maximum at 470 nm, and thus the reaction can be readily measured photometrically. The enzyme was extracted by 10 mM phosphate buffer (pH=7.0), and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. The reaction was started by addition of the enzyme extract at 25°C and was followed for 3 min. The enzyme unit was calculated as enzyme protein required for the formation of 1 µM tetraguaiacol for 1 min.

Polyphenoloxidase: Polyphenoloxidase (EC 1.10.31) activity was assayed spectrophotometrically by monitoring the absorbance at 430 nm [31]. The enzyme was extracted by 10 mM phosphate buffer (pH=7.0), and assayed in a solution contained 200 mM phosphate buffer and 20 mM pyrogallol. The reaction was started by addition of the enzyme extract at 28°C and was followed for 3 min. The enzyme unit was calculated as enzyme protein required for color change for 1 min.

Discontinuous sodium dodecyl-sulfate polyacrylamid gel electrophoresis (SDS-PAGE) was performed according to Laemmli [41] with 12 % acrylamide gels and 50 µg of protein per lane was loaded. For detection of proteins, gel was stained with 0.03 % Coomassie Brilliant Blue G250.

Native gel electrophoresis (non-denaturing conditions) for isoenzyme assay was carried out according to modified method of Davis [22] with a 10% acrylamide gel at 4°C and 100 µg of protein per lane was loaded. A vertical electrophoresis apparatus (*model LKB, Bromma, Stockholm, Sweden*) was used. The electrophoresis run was carried out with 120 mV (30 mA) per plate towards the cathode. Determination of protein in tissue extracts for calculation of enzyme activities were carried out according to Bradford [5].

Experiments were conducted in a randomized complete block design using four replications. Statistical analyses were carried out using Sigma Stat software (3.02).

Results

Plants Growth and Accumulation of Cu and Zn in Shoots and Roots

Toxic concentrations of Zn did not affect shoot and root growth of *S. monatum* based on shoot and root dry weight. Root growth of this species was rather promoted up to 51% in response to 100 µM Zn and shoot growth was promoted at lower concentrations (50 µM) and was

not affected at high (100 μM) concentrations of Zn (Table 1).

Zn accumulation in shoot was higher in *D. glomerata* than *S. monatum*. In *D. glomerata* up to 0.7 mg g^{-1} (d.w.) and in roots 4 mg g^{-1} (d.w.) Zn was accumulated in plants treated with 100 μM Zn. Zinc accumulation in dry matter of shoot and root in *S. monatum* was approximately 50% less than that in *D. glomerata* (Table 1).

Copper toxicity effect was also different between two species studied. *C. intybus* showed a high sensitivity to toxic concentrations of Cu in both shoot and root but *A. echinops* demonstrated a relatively high tolerance. The growth reduction in shoot was 81% and 58% and in root 70% and 49% in *C. intybus* and *A. echinops* respectively (Table 2).

Copper accumulation in shoot and root was also different between two species. A higheraccumulation was observed in shoot and roots of *C. intybus* than *A. echinops*. Copper accumulation was much higher in roots than shoots, so that up to 17 (*C. intybus*) and 10 (*A. echinops*) mg Cu per g (d.w.) of root was accumulated in plants treated with 100 μM Cu (Table 2).

As expected, uptake and transport of Zn increased with increasing Zn concentrations in medium. The percentage of transport of total Zn taken up was similar between two species (data not shown). However, the absolute values of Zn taken up and transported into shoot were higher in *D. glomerata* than *S. monatum* (Fig. 1).

A clear difference was shown between two species studied in the absolute values of Cu taken up and transported into shoot. Copper uptake and transport were higher in *C.intybus* than *A. echinops.* Comparison between two heavy metals showed that, the percentage of transport of Cu into shoot was much higher than that of Zn. The highest Zn transport was 25%, (*e.g.* in *D. glomerata*), while the transported Cu into shoot was up to 75% (in *C. intybus*) at the same concentration (100 μM) of metals (Fig. 2).

Accumulation of Organic Acids, Amino Acids and Amides

Malic and citric acid was not found in detectable amounts in shoot of *S. monatum*. In *D. glomerata* a relatively high endogenous organic acids was found, but no change in response to Zn treatment was observed. In response to Zn treatment, malate of roots rather decreased and citrate level decreased (*D. glomerata*) or remained unchanged (*S. monatum*) (Table 3).

Endogenous malate concentration was much higher in *A. echinops* than *C. intybus* in all Cu treatments. A slight reduction of malate in shoot and increase in roots in response to Cu treatments was observed in *A. echinops* treated with 100 μM Cu. Citrate was not detected in *A. echinops*, neither in shoot nor in roots (Table 4).

Histidine and asparagines were not detectable by TLC. Detectable amounts of glutamine were observed in *S. monatum* and only cysteine content responded to Zn treatments, which either increased in *D. glomerata* or decreased in *S. monatum* (Table 5).

Histidine and asparagine were not found in *C. intybus* and glutamine was not found in *A. echinops*. Similar to Zn treatments, only cysteine content responded to Cu treatment in *C. intybus* but not in *A. echinops*. However, the endogenous content of cysteine was much higher in *A. echinops* than *C. intybus*. The cysteine content increased in response to 50 μM Cu and decreased with increasing Cu concentration in the medium (Table 6).

Measured Cysteine concentration showed that in *A. echinops* a significant increase in response to 100 μM Cu occurred in shoots. In contrast, Cu treatment of roots, did not result the same response as shoots (Table 7).

Alteration in Proteins and Enzymes Activity in Response to Cu Treatment

Analysis of proteins by SDS-PAGE did not showed any significant changes. According to both spectrophotometric and PAGE analysis of enzymes, activity of catalase and polyphenol oxidase [unit mg^{-1}(protein)] did not change under Cu treatment in both of species. However, peroxidase activity [unit mg^{-1}(protein)] decreased under Cu toxicity. Reduction of peroxidase activity was much higher in *C. intybus* than *A. echinops* in both shoot and root. In *C. intybus* the reduction of peroxidase activity in comparison with control was 47% and 39% and in *A. echinops* was 10% and 28% in shoot and root respectively (Table 8).

Discussion

Growth and Accumulation of Zn

A clear difference between two species studied was observed in response to toxic concentrations of Zn. *S. monatum* showed to be a high Zn tolerant species, but the shoot and root growth of *D. glomerata* were inhibited in Zn concentration at 25 μM. Growth improvement in Zn concentrations of 100 μM for root and at 50 μM for shoot, indicated that *S. monatum* could be classified as a high tolerant species to Zn. For other species such as *Arabidopsis halleri*, plants growth was

Table 1. Growth and concentration of Zn in Shoot and roots of *Dactylis glomerata* and *Secale monatum* grown at toxic levels of this metal in the nutrient solution. Values in each column within each plant species followed by the same letter are not significantly different (P<0.05)

	Zn (μM)	Dry weight [mg (plant)$^{-1}$]		Zn concentration [μg g^{-1} (d.w.)]	
		Shoot	Root	Shoot	Root
D. glomerata	0	25.2±5.0^{a}	8.7±2.1^{a}	165±43 ^{b}	457±71 ^{b}
	25	20.4±1.1^{ab}	6.8±1.2^{a}	632±146 ^{a}	2586±990 ^{ab}
	50	16.9±3.6^{b}	7.3±2.0^{a}	619±154 ^{a}	3192±1949 ^{ab}
	75	16.0±3.7^{b}	6.4±1.9^{a}	709±162 ^{a}	3963±1269 ^{a}
	100	16.2±2.4^{b}	6.5±2.1^{a}	548±413 ^{a}	4039±2457 ^{a}
S. monatum	0	43.7±3.9^{b}	10.1±1.6^{c}	88±7 ^{c}	850±140 ^{b}
	25	52.8±3.3^{ab}	17.2±5.5^{ab}	305±38 ^{ab}	1289±99 ^{ab}
	50	55.0±6.5^{a}	18.2±1.2^{a}	280±51 ^{b}	1640±127 ^{ab}
	75	45.1±7.4^{ab}	12.8±1.2^{bc}	358±17 ^{a}	1898±301 ^{a}
	100	43.5±6.9^{b}	15.3±2.9^{abc}	338±35 ^{ab}	2061±658 ^{a}

Table 2. Growth and concentration of Cu in Shoot and roots of *Chicorium intybus* and *Astragalus echinops* grown at toxic levels of this metal in the nutrient solution. Values in each column within each plant species followed by the same letter are not significantly different (P<0.05)

	Zn (μM)	Dry weight [mg (plant)$^{-1}$]		Cu concentration [μg g^{-1} (d.w.)]	
		Shoot	Root	Shoot	Root
C. intybus	0	125.0±40.1^{a}	44.3±7.9^{b}	748±102 ^{c}	2078±523 ^{b}
	25	113.2±23.6^{a}	62.3±3.1^{a}	1598±458 ^{bc}	5289±313 ^{b}
	50	135.1±30.6^{a}	65.7±14.1^{a}	2070±902 ^{ab}	7398±994 ^{b}
	75	73.7±17.3^{b}	49.0±8.6^{b}	2160±168 ^{ab}	14721±742 ^{ab}
	100	23.7±7.3^{c}	13.3±2.1^{c}	3159±144 ^{a}	17642±8471 ^{a}
A. echinops	0	27.2±7.5^{a}	56.2±3.5^{a}	18±1 ^{c}	117±11 ^{e}
	25	13.5±4.6^{b}	49.0±4.2^{b}	29±4 ^{c}	2642±112 ^{d}
	50	8.3±1.0^{b}	52.8±1.3^{a}	552±43 ^{b}	5783±612 ^{c}
	75	6.2±2.3^{b}	23.9±1.8^{b}	616±28 ^{b}	9374±111 ^{b}
	100	11.3±2.9^{b}	28.6±2.3^{b}	882±61 ^{a}	10748±113 ^{a}

also not negatively affected by 100 μM Zn [72].

A high sensitivity to Zn toxicity was associated with high Zn accumulation in shoot and root. The Zn concentration in *D. glomerata* was an order of magnitude higher than *S. monatum.* Therefore, *D. glomerata* could be considered as an includer plant with a high sensitivity to Zn toxicity. Zn hyperaccumulators are defined as being able to accumulate more than 10,000 μg Zn in the above ground parts on a dry weight basis [9]. The Zn concentration of leaves in samples of *S. monatum* collected from Zn rich soils of North West Iran was in the range of 100-300 μg g^{-1} DW, which was similar to Zn concentrations of plants in the present hydroponic experiment. Therefore, *S. monatum* could be ranked as an includer, tolerant but non-accumulator species. Presence of *D. glomerata* on Zn-rich soils, was restricted to marginal areas [30], obviously because of a high sensitivity to Zn toxicity as was also documented in this hydroponic experiment.

Growth and Accumulation of Cu

Like Zn, two species studied showed a clear difference in growth response to toxic concentrations of Cu. *C. intybus* demonstrated a high sensitivity, particularly at high Cu concentrations. A high susceptibility to Cu in *C. intybus* was associated with a high Cu accumulation in shoot and roots. It means that, *C.*

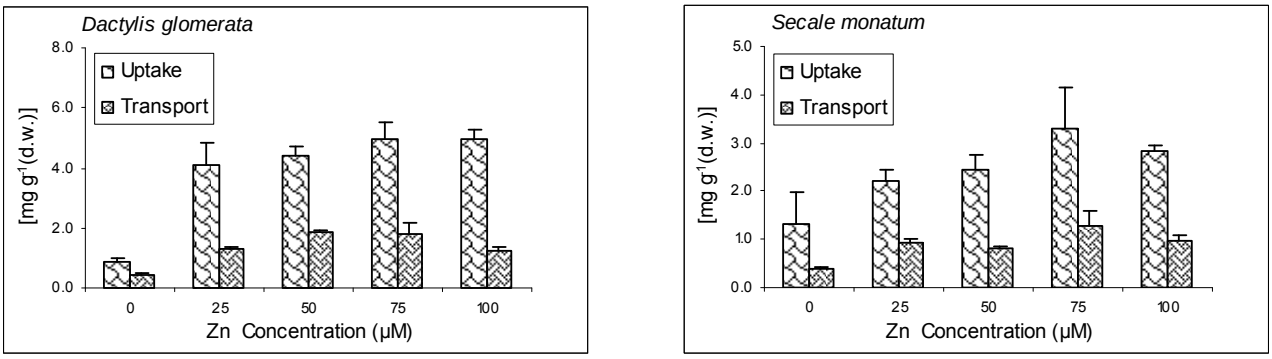

Figure 1. Uptake [mg g^{-1} (d.w.)] and transport [mg g^{-1} (d.w.)] of Zn in *D. glomerata* and *S. monatum* in response to toxic concentrations of Zn in medium.

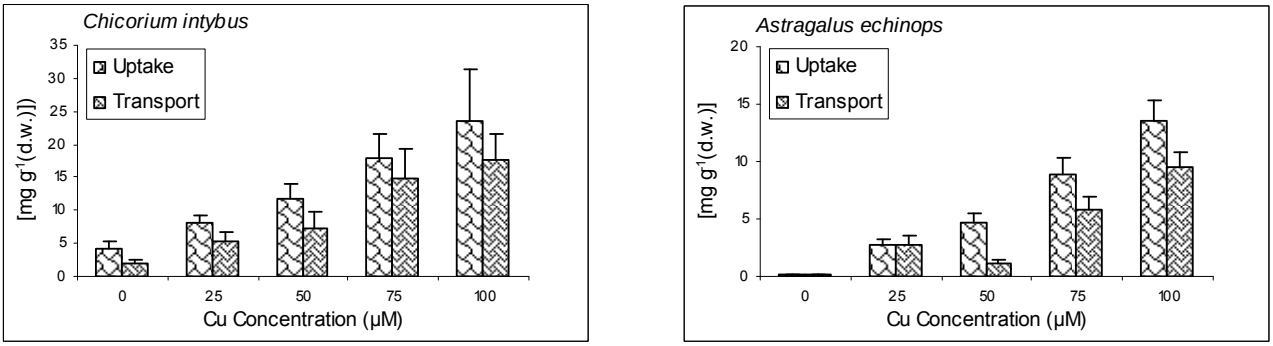

Figure 2. Uptake [mg g^{-1} (d.w.)] and transport [mg g^{-1} (d.w.)] of Cu in *C. intybus* and *A. echinops* in response to toxic concentrations of Cu in medium.

intybus is an includer and sensitive species to Cu toxicity. The Cu concentration in samples of *A. echinops* collected from Cu rich soils of North West Iran was in the range of 50-500 µg g^{-1} DW, which was similar to Cu concentrations of plants in the present hydroponic experiment. Therefore, according to the definition of Cu hyperaccumulator and accumulator species [9], *A. echinops* could be ranked as an accumulator species. *C. intybus* was absent from these highly Cu-rich areas [30], most likely because of a high susceptibility to Cu toxicity.

Accumulation of Potential Chelating Molecules

In roots of *S. monatum* a high endogenous malate and citrate concentration may be one of the causes of higher tolerance of this species to Zn, considering particularly that, root growth of this species improved in response to all toxic Zn concentrations applied. In a study on tobacco suspension cells, metal-dependent stimulation of malic acid and citric acid was not observed. However, it was proved that, even at very high growth-inhibiting levels of Zn, sufficient organic acid is present in the vacuole [39].

However, in shoot, organic acids had no determining role in tolerance of *S. monatum*. Though low concentration of organic acids, concomitant with high tolerance, an improvement in growth was observed at 50 µM Zn concentration.

In contrast to organic acids in roots, accumulation of amino acids was not in association with growth response of two species studied either in root or shoot. Cysteine concentration did not only increase but a reduction was also detected in shoots of *S. monatum*. Other investigations showed that, low molecular weight compounds have no distinct effect on detoxicfication of Zn [33]. One of the important mechanisms for Zn detoxification in Zn tolerant populations of *Silene vulgaris*, is the sequestration of Zn in vacuoles [69]. On the other hand, the difference between hyperaccumulator and non-accumulator species of genus *Thlaspi* was attributed to the higher V_{max} values in the

Table 3. Detection of organic acids by TLC in tissue extract of *Dactylis glomerata* and *Secale monatum* grown at toxic levels of Zn in the nutrient solution

	Zn (µM)	Shoot		Root	
		Malic acid	Citric acid	Malic acid	Citric acid
D. glomerata	0	++	++	++	++
	50	++	++	+	+
	100	++	++	+	+
S. monatum	0	–	–	+++	++
	50	–	–	++	++
	100	–	–	++	++

Key: The number of + signs is correlated with the concentration.

Table 4. Detection of organic acids by TLC in tissue extract of *Chicorium intybus* and *Astragalus echinops* grown at toxic levels of Cu in the nutrient solution

	Cu (µM)	Shoot		Root	
		Malic acid	Citric acid	Malic acid	Citric acid
C. intybus	0	+	++	+	+
	50	+	+	+	+
	100	+	+	+	+
A. echinops	0	+++	–	++	–
	50	+++	–	+++	–
	100	++	–	+++	–

Key: The number of + signs is correlated with the concentration.

Table 5. Detection of amino acids by TLC in tissue extract of *Dactylis glomerata* and *Secale monatum* grown at toxic levels of Zn in the nutrient solution

	Zn (µM)	Shoot				Root			
		Cysteine	Histidine	Glutamine	Asparagine	Cysteine	Histidine	Glutamine	Asparagine
D. glomerata	0	+	–	+	–	+	–	+	–
	50	++	–	+	–	+	–	+	–
	100	+	–	+	–	+	–	+	–
S. monatum	0	++	–	–	–	+	–	–	–
	50	+	–	–	–	+	–	–	–
	100	+	–	–	–	+	–	–	–

Key: The number of + signs is correlated with the concentration.

Table 6. Detection of the amino acids by TLC in tissue extract of *Chicorium intybus* and *Astragalus echinops* grown at toxic levels of Cu in the nutrient solution

	Cu (µM)	Shoot				Root			
		Cysteine	Histidine	Glutamine	Asparagine	Cysteine	Histidine	Glutamine	Asparagine
C. intybus	0	++	–	+	–	+	–	+	–
	50	+++	–	+	–	+	–	+	–
	100	+	–	+	–	+	–	+	–
A. echinops	0	+++	+	–	+	+	+	–	+
	50	+++	+	–	+	+	+	–	+
	100	+++	+	–	+	+	+	–	+

Key: The number of + signs is correlated with the concentration.

Table 7. The concentration of cysteine [μg g^{-1}(f.w.)] in tissue extract of *Chicorium intybus* and *Astragalus echinops* grown at toxic levels of Cu in the nutrient solution. Values in each column followed by the same letter are not significantly different (P<0.05)

Cu (μM)	*Chicorium intybus*		*Astragalus echinops*	
	Shoot	Root	Shoot	Root
0	313±112 [a]	109±28 [a]	703±46 [b]	262±67[a]
50	370±93 [a]	87±12 [a]	127±57 [c]	9±1[c]
100	264±129 [a]	104±21 [a]	903±35 [a]	133±2[b]

Table 8. Activity of peroxidase (POD), catalase (CAT) and polyphenoloxidase (PPO) [unit mg^{-1}(protein)] in tissue extract of *Chicorium intybus* and *Astragalus echinops* grown at toxic levels of Cu in the nutrient solution. Values in each column followed by the same letter are not significantly different (P<0.05)

	Cu (μM)	*Chicorium intybus*		*Astragalus echinops*	
		Shoot	Root	Shoot	Root
POD	0	87.31±0.87[a]	3.28±0.06[a]	109.22±1.2[a]	2.85±0.02[a]
	50	46.54±1.38[b]	2.01±0.07[b]	98.03±1.9[b]	2.04±0.01[b]
CAT	0	1014.6±7.35[a]	1230.7±3.2[a]	995.1±12.11[a]	1011.4±5.8[a]
	50	1071.1±56.04 [a]	1275.5±16.0[a]	983.7±10.91 [a]	1041.8±12.9 [a]
PPO	0	5.85±0.19[a]	0.34±0.02[a]	12.13±0.37[a]	0.48±0.02[a]
	50	5.99±0.41[a]	0.33±0.1[a]	11.97±0.24[a]	0.52±0.02[a]

hyperaccumulator species [42]. We observed a clear difference in Zn uptake and transport in this study.

A considerable difference in malate accumulation in shoot and root between two species studied for Cu toxicity was observed. This difference was most likely the main cause of Cu tolerance in *A. echinops* compared to *C. intybus*. It was shown that, malate could act as a potent chelating molecule for Ni in many plant species [56,57]. Although there is no report on Cu chelation by malate, regarding stability constants [34], it is possible that excess Cu was chelated by malate in *A. echinops*. Although we did not observe inductive effect of Cu on malate accumulation in *A. echinops*, a constitutive higher malate concentration in *A. echinops*, could be due to Cu tolerance in this species.

Like malate, the concentration of cysteine was also higher in *A. echinops* in comparison with *C. intybus*, documented by both TLC and spectrophotometry. On the other hand, histidine was absent in shoot and root extract of *C. intybus*, while it was detected in trace amount by TLC in *A. echinops*. Although inductive effect of Cu on the histidine amounts was not observed, its presence in detectable amounts could be another reason for the tolerance of *A. echinops* to Cu toxicity. Chelating effect of histidine for Ni, so called histidine effect is well documented [34,38].

Induction of oxidative stress is one of the main causes of heavy metal toxicity in plants. Reduction in the activity of antioxidant enzymes including peroxidase was reported under Cr [61] Cd [16] Ni [3] and Cu [36] toxicity. The extent of changes in the activity of antioxidant enzymes under metal toxicity could be one of the most important factors determining responses of two tested species to Cu toxicity. Accordingly, in this work, a higher inhibition in the activity of peroxidase in *C. intybus* was in association with higher susceptibility to excess Cu. Therefore, reduction of activity of enzymes could be assumed as a biochemical symptom of Cu, which is strongly expressed in sensitive rather tolerant species. However, the activity of catalase and polyphenol oxidase remained unchanged under Cu treatment.

Guaiacol peroxidase, which was assayed in this work, is involved in the lignification of cell wall. A lower lignification capacity of cell wall under Cu toxicity could be one of the reasons for stronger root growth inhibition in *C. intybus*. The Cu induced membrane damages and leakage of solutes from roots is well documented [50]. Therefore, lignification could prevent leakage of solutes from roots. Induction of lignification under excess Cu stress was reported by other investigators [13,14,25]. A possible role for lignification in establishing an apoplastic transport barrier in roots was hypothesized under Cu and Zn toxicity [20].

There have been some physiological studies on the

uptake and translocation of metals by metal hyper-accumulators, but a few investigations have been published to identify the mechanisms of metal tolerance [18]. Studies on the mechanisms involved in Zn hyperaccumulation showed that, the Zn hyperaccumulation is not associated with rhizosphere acidification [37,48], but with a Zn uptake velocity (Vmax) 4-5 times more than a non-accumulating species [42]. This plant also possesses highly efficient mechanisms for transport of Zn from roots to shoots [43,65].

References

1. Ali N.A., Bernal M.P., and Ater M. Tolerance and Bioaccumulation of Copper in *Phragmites australis* and *Zea mays*. *Plant Soil*, **239**: 103-111 (2002).
2. Baker A.J.M., Mc Grath S.P., Reeves R.D., and Smith J.A.C. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry N. and Banuelos G. (Eds.), *Phytoremediation of Contaminated Soil and Water*. Lewis Publishers, Boca Raton, FL, pp. 85-107 (2000).
3. Boominathan R. and Doran P.M. Ni-induced oxidative stress in roots of the Ni hyperaccumulator, *Alyssum bertolonii*. *New Phytol*., **156**: 205-215 (2002).
4. Boominathan R., and Doran P.M. Organic acid complexation, heavy metal distribution and the effect of ATPase inhibition in hairy roots of hyperaccumulator plant species. *J. Biotechnol.,* **101**: 131-146 (2003).
5. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72**: 248-254 (1976).
6. Brooks R.R., Reeves R.D., Morrison R.S., and Malaisse F. Hyperaccumulation of copper and cobalt, A review. *Bulletin de la Société Royale Botanique de Belgique*, **113**: 166-172 (1980).
7. Brooks R.R., and Malaisse F. *The Heavy Metal Tolerant Flora of Southcentral Africa*. Balkema, Rotterdam, p. 199 (1985).
8. Brooks R.R., Baker A.J.M., and Malaisse F. Copper flowers. *National Geographic Research,* **8**: 338-351 (1992).
9. Brooks R.R. Geobotany and hyperaccumulators. In: Brooks, R.R. (Ed.), *Plants that Hyperaccumulate Heavy Metals*, CAB International, USA, pp. 55-94 (1998).
10. Brune A., Urbach W., and Dietz K.-J. Compartmentaion and transport of zinc in barley primary leaves as basic mechanisms involved in Zn tolerance. *Plant Cell Environ.,* **17**: 153-162 (1994).
11. Cakmak I., Sari N., Marschner H., Kalayci M., Yilmaz A., Eker S., and Gülüt K.Y. Dry matter production and distribution of zinc in bread and durum wheat genotypes differing in zinc efficiency. *Plant Soil*, **180**: 173-181 (1996).
12. Chance B., and Maehly A.C. Assay of catalases and peroxidases. *Methods in Enzymology*, **2**: 764-775 (1955).
13. Chaoui A., Brahim J., and El Ferjiani E. Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. *J. Plant Physiol*., **161**: 1225-1234 (2004).
14. Chaoui A., and El Ferjani E. Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. *C. R. Biologies*, **328**: 23-31 (2005).
15. Chardonnens A.N., Koevoets P.L.M., Zanten A.V., Schat H., and Verkleij J.A.C. Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. *Plant Physiol*., **120**: 779-785 (1999).
16. Chen Y.X., He Y.F., Luo Y.M., Yu Y.L., Lin Q., and Wong M.H. Physiological mechanism of plant roots exposed to cadmium. *Chemosphere,* **50**: 789-793 (2003).
17. Clarkson D.T. and Lüttge U. Mineral nutrition: divalent cations, transport and compartmetation. *Progress in Botany,* **51**: 93-11 (1989).
18. Clemens S. Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*, **212**: 475-486 (2001).
19. Cobbett C.S. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol*., **123**: 825-832 (2000).
20. Cuypers A.N.N., Vangronsveld J., and Clijsters H. Peroxidases in roots and primary leaves of Phaseolus vulgaris copper and zinc pytotoxicity: a comparison. *J. Plant Physiol*., **159** (8): 869-876 (2002).
21. Dannel F., Pfeffer H., and Marschner H. Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. *J. Plant Physiol*. **146**: 273-278 (1995).
22. Davis B.J. Disc electrophpresis. II. Method and Application to Human Serum Proteins. *Ann. New York Acad. Sci*., **121**: 404-427 (1964).
23. Delhaize E., and Ryan P.R. Aluminum toxicity and tolerance in plants. *Plant Physiol*. **107**: 315-321 (1995).
24. De Vos Ch.H.R., Vonk M.J., Vooijs R., and Schat H. Glutathione depletion due to copper-induced phyto-chelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiol*., **98**: 853-858 (1992).
25. Díaz J., Benal A., Pomar F., and Merino F. Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annuum* L.) seedlings in response to copper stress and its relation to lignification. *Plant Sci*., **161**: 179-188 (2001).
26. Fernandes J.C. and Henriques F.S. Biochemical, Physiological and Structural Effects of Excess Copper in Plants. *Bot. Rev*., **57**: 246-273 (1991).
27. Godbold D.L., Horst W.J., Collons J.C., Thurmann P.A., and Marschner H. Accumulation of zinc and organic acids in roots of zinc tolerant and non-tolerant ecotypes of *Deschampsia caespitosa*. *J. Plant Physiol*. **116**: 59-69 (1984).
28. Gaitonde M.K. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J*., **104**: 627-633 (1967).
29. Ha S.B., Smith A.P., Howden R., Dietrich W.M., Bugg S.O., Conneli M.J., Goldsbrough P.B., and Cobbett C.S. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell*, **11**: 1153-1163 (1999).

30. Hajiboland R. Flora of heavy metal rich soils in NW Iran and introduction of some accumulator species. In: Proceeding Book of 10th Iranian Biology Conference, Univ. Tehran, Iran (2002).
31. Halder J., Tamuli P., and Bhaduri A.N. Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camelia sinensis*). *J. Nutr. Biochem.*, **9**: 75-80 (1998).
32. Harmens H., Den Hartog P.R., Ten Bookum W.M., and Verkleij J.A.C. Increased zinc tolerance in *Silene vulgaris* is not due to increased production of phytochelatins. *Plant Physiol*., **103**: 1305-1309 (1993).
33. Harmens H., Koevoets P.L.M., Verkleij J.A.C., and Ernst W.H.O. The role of low molecular weight organic acids in the mechanism of increased zinc tolerance in *Silene vulgaris* (Moench) Garcke. *New Phytol*., **126**: 615-621(1994).
34. Homer F.A., Reeves R.D., and Brooks R.R. The possible involvement of amino acids in nickel chelation in some nickel-accumulating plants. *Current Topics in Phytochemistry*, **14**: 321-33 (1997).
35. Jarvis S.C. and Whitehead D.C. The Influence of Some Soil and Plant Factors on the Concentration of Copper in Perennial Ryegrass. *Plant Soil*, **60**: 275-286 (1981).
36. Jouili H. and Ferjani E.E. Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess. *C.R. Biologies*, **326**: 639-644 (2003).
37. Knight K., Zhao F.J., Mc Grath S.P., and Shen Z.G. Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant Soil*, **197**: 71-78 (1997).
38. Krämer U., Cotter-Howells J.D., Charnock J.M., Baker A.J.M., and Smith J.A.C Free histidine as a metal chelator in plants that accumulate nickel. *Nature*, **379**: 635-638 (1996).
39. Krotz R.M., Evangelou B.P., and Wagner G.J. Relationship between cadmium, zinc, Cd-peptide, and organic acid in tobacco suspension cells. *Plant Physiol.*, **91**: 780-787 (1989).
40. Küpper H., Zhao F.J., and Mc Grath S.P. Cellular compartmentation of zinc in leaves of the hyperaccumulator of *Thlaspi caerulescens. Plant Physiol.*, **119**: 305-311 (1999).
41. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-695 (1970).
42. Lasat M.M., Baker A.J.M., and Kochian L.V. Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiol*., **112**: 1715-1722 (1996).
43. Lasat M.M., Baker A.J.M., and Kochian L.V. Altered Zn compartmentaion in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Ibid*., **118**: 875-883 (1998).
44. Lasat M.M., Pence N.S., Garvin D.F., Ebbs S.D., and Kochian L.V. Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescence*. *J. Exp. Bot*., **51**(342): 71-79 (2000).
45. Lockwood, G.B., Brain K.R., and Turner T.D. Simultaneous estimation of sterol and steroidal sapogenin in plant extracts by densitometric thin-layer chromatography. *Journal of Chromatography,* **95**: 250-253 (1974).
46. Lück H. In: Bergmeyer H.U. (Ed.), *Methods of Enzymatic Analysis*, VCH, Weinheim, Germany, pp. 885-894 (1962).
47. Mathys W. The role of malate, oxalate and mustard oil glucosoids in the evolution of zinc resistance in herbag plants. *Physiol. Plant*., **40**: 130-136 (1977).
48. Mendoza-Cózatl D.G. and Moreno-Sánchez R. Control of glutathione and phytochelatin synthesis under cadmium stress. Pathways modeling for plants. *J. Theor. Biology*, **238**: 919-936 (2006).
49. Mc Grath S.P., Shen Z.G., and Zhao F.J. Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant Soil*, **188**: 153-159 (1997).
50. Murphy A.S., Eisinger W.R., Shaff J.E., Kochian L.V., Taiz L. Early copper-induced leakage of K^+ from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. *Plant Physiol*., **121**: 1375-1382 (1999).
51. Neumann G., Massonneau A., and Römheld V. Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta*, **2058**: 373-382 (1999).
52. Ouzounidou G. Changes of Photosynthetic Activities in Leaves as a Result of Cu-Treatment: Dose-Response Relation in *Silene* and *Thlaspi*. *Photosynthetica*, **29**: 455-462 (1993).
53. Ouzounidou G. Copper-induced changes on growth, metal content and photosynthetic functions of *Alyssum monatum* L. plants. *Environ. Exp. Bot*., **34**: 165-172 (1994a).
54. Ouzounidou G. Root growth and pigment composition in relationship to element uptake in *Silene compacta* plants treated with copper. *J. Plant Nutr*., **17**: 933-943 (1994b).
55. Oven M., Grill E., Goldhirsh A.G., Kutchan T.M., and Zenk M.H. Increase of free cysteine and citric acid in plant cells exposed to cobalt ions. *Phytochemistry*, **60**: 467-474 (2002).
56. Pancaro L., Pelosi P., Vergnano gambi O., and Galoppini C. Further contribution on the relationship between nickel and malic and malonic acids in Alyssum. *Gionale Botanica Italiano*, **112**: 282-283 (1978).
57. Pelosi P., Fiorentini R., and Galoppini C. On the nature of nickel compounds in *Alyssum bertolonii* Desv. *Agricultural and Biological Italiana*, **29**: 1-5 (1976).
58. Pich A. and Scholz I. Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill.): nicotianamine-stimulated copper transport in xylem. *J. Exp. Bot*., **47**: 41-47 (1996).
59. Poulter A., Collin H.A., Thurman D.A., and Hardwick K. The role of cell wall in the mechanism of lead and zinc tolerance in *Anthoxanthum odoratum* L. *Plant Sci*., **42**: 61-66 (1985).
60. Prasad M.N.V., Malec P., Waloszek A., Bojko M., and Strzalka K. Physiological Responses of *Lemna trisulca* L.

(Duckweed) to Cadmium and Copper Bioaccumulation. *Plant Sci*., **161**: 881-889 (2001).
61. Rai V., Vajpayee P., Singh S.N., and Mehrotra S. Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, praline level and eugenol content of *Ocimum tenuiflorum* L. *Ibid*., **167**: 1159-1169 (2004).
62. Rauser W.E. Structure and function of metal chelators produced by plants-the case of organic acids, amino acids, phytin and metallothioneins. *Cell Biochemistry and Biophysics*, **31**: 19-48 (1999).
63. Salt D.E., Prince R.C., Baker A.J.M., Raskin I., and Pickering J.I. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environ. Sci. Technol*., **33**: 713-717 (1999).
64. Sagner S., Kneer R., Wanner G., Cosson J.-P., Dues-Neumann B., and Zenk M.H. Hyperaccumulation, complexation and distribution of nickel in *Sebertia accuminata*. *Phytochemistry*, **47**: 339-347 (1998).
65. Shen Z.G., Zhao F.J., and Mc Grath S.P. Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non-hyperaccumulator *Thlaspi ochroleucum. Plant Cell Environ*., **20**: 898-906 (1997).
66. Simon L.M., Fatrai Z., Jones D.E., and Matkovics B. Study of peroxide metabolism enzymes during the development of *Phaseolus vulgaris*. *Biochem. Physiol. Pflanzen (BPP)*, **166**: 387-392 (1974).
67. Vázquez M.D., Barceló J., Poschenrieder C.H., Mádico J.Ch., Hatton P., Baker A.J.M., and Cope G.H. Localization of zinc and cadmium in *Thlaspi caerulescens* (Brassicaceae), a metallophte that can hyperaccumulate both metals. *J. Plant Physiol*., **140**: 350-355 (1992).
68. Vázquez M.D., Poschenrieder C.H., Barceló J., Baker A.J.M., Hatton P., and Cope G.H. Compartmentation of zinc in roots and leaves of zinc hyperaccumulator of *Thlaspi caerulescens* J&C Presl. *Botanica Acta*, **107**: 243-250 (1994).
69. Verkleij J.A.C., Koevoets P.L.M., Blake-Kalff M.A., and Chardonnens A.G.N. Evidence for an important role of the tonoplast in the mechanism of naturally selected zinc tolerance in *Silene vulgaris*. *J. Plant Physiol*., **153**: 188-191 (1998).
70. Wagner G.J. Accumulation of cadmium in crop plants and its consequences to human health. *Adv. Agron*., **51**: 173-212 (1993).
71. Wang J., Evangelou B.P., Nielsen M.T., and Wagner G.J. Computer-simulated evaluation of possible mechanisms for sequestering metal ion activity in plant vacuoles. II. Zinc. *Plant Physiol*., **99**: 651-626 (1992).
72. Zhao F.J., Lombi E., Breedon T., and Mc Grath S.P. Zinc hyperaccumulation and cellular distribution in *Arabidopsis helleri*. *Plant Cell Environ*., **23**(5): 507-514 (2000).