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ScienceDirect
Chemosphere 67 (2007) 2138–2147
CHEMOSPHERE
www.elsevier.com/locate/chemosphere

The copper tolerance strategies and the role of antioxidative enzymes in three plant species grown on copper mine

Massod Mashhadi Akbar Boojar *, Faranak Goodarzi

Department of Biology, University of Tarbiat Moalem, No. 49, Dr. Mofateh Avenue, Tehran, P.O. Box 15614, Iran

Received 16 December 2005; received in revised form 9 December 2006; accepted 19 December 2006
Available online 20 February 2007

Abstract

This study was undertaken to identify the strategies and the status of antioxidant enzyme activities involved in three plant species tolerance against Cu-toxicity in copper mine. The following methods were used for evaluations in three wild type species; *Datura stramonium*, *Malva sylvestris* and *Chenopodium ambrosioides*. The level of chlorophyll and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) by spectrometry, malondialdehyde (MDA) and dityrosine by HPLC and the levels of Cu in tissues and soils by atomic absorption spectrometry (AAS).

Analysis showed that total and available copper were at toxic levels for plants growing on contaminated soil (zone 1). However, there were not any visual and conspicuous symptoms of Cu toxicity in plant species. Among three species, excess copper was transferred only into the *D. stramonium* and *C. ambrosioides* tissues. The *C. ambrosioides* accumulated Cu in roots and then in leaves, in which the leaves chloroplasts stored Cu around two times of vacuoles. In *D. stramonium* most of Cu was accumulated in leaves in which the storage rate in vacuoles and chloroplasts were 42% and 8%, respectively. In zone 1, the chlorophyll levels increased significantly in leaves of *C. ambrosioides* with respect to the same plant growing on uncontaminated soil (zone 2). There was insignificant decrease in chlorophyll content of *D. stramonium* leaves, collected from zone 1 with respect to zone 2. The *D. stramonium* and *C. ambrosioides* in zone 1, both revealed significant increase in their tissues antioxidant enzyme activities in comparison with the same samples of zone 2. There was significant elevation in oxidative damage biomarkers; MDA and dityrosine, when the aerial parts of *D. stramonium* in zone 1 were compared with the same parts of zone 2.

We concluded that there were different tolerance strategies in studied plant species that protected them against copper toxicity. In *M. sylvestris*, exclusion of Cu from the roots or its stabilization in the soil restricted Cu toxicity effects. On the other hand *D. stramonium* and *C. ambrosioides*, elevated their antioxidative enzyme activities in response to cu-toxicity. In addition, the species *D. stramonium* accumulated excess of Cu in leaves vacuoles.

Keywords: Copper mine; Antioxidative enzyme; Malondialdehyde; Dityrosine; Vacuoles; Chlorophyll; Plant tolerance

1. Introduction

Copper (Cu) is both a micronutrient for plants and a heavy metal capable of stress induction (Thomas et al., 1998). This trace element plays important roles in CO_2 assimilation, ATP synthesis and is a component of various proteins and particularly those involved in both the photosynthetic (plastocyanin) and the respiratory (cytochrome oxidase) electron transport chain (Demirevska-kepova et al., 2004). The uptake of the copper from soil by plants depends on the ability of the plants to transfer the metal across the soil–root interface and the total amount of Cu present in the soil (Agata and Ernest, 1998). In recent decades, enhanced industrial and mining activities have contributed to the increasing occurrence of heavy metals including copper in ecosystems. Copper is a widespread

* Corresponding author. Tel.: +98 21 88102304/22736470; fax: +98 21 88848940.
E-mail address: aboojar@yahoo.com (M.M.A. Boojar).

0045-6535/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2006.12.071

contaminant originating from different human activities including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface. Excessive of Cu in soil, plays cytotoxic role, induces stress and can unfavorably cause injury and symptoms to plant including growth retardation and leaf chlorosis (Baker and Proctor, 1990; Waldermar et al., 1994; Lewis et al., 2001). Accordingly, Cu toxicity has important implication for effects of copper mines ecosystems prone to Cu stress in which the role of oxidative stress and reactive oxygen species (ROS) production may be involved (Stadtman and Oliver, 1991). Under Cu toxicity, excess copper is an efficient generator of ROS in Fenton-type reactions, leading to disturbance of metabolic pathways and macromolecules damages (Hegedus et al., 2001). ROS are generally very reactive molecules possessing an unpaired electron and under normal conditions their levels in plant cells are under tightly control by scavenging system. However, when ROS are not adequately removed, an effect termed "oxidative stress" may result. Excess ROS formed within cells, can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and even DNA, creating oxidative injury that results in a reduction of plant growth and development (Ogawa and Iwabuchi, 2001; Hernandez-Jimenez et al., 2002).

Because the toxic intermediates and ROS are short-lived and difficult to measure directly, an alternative approach for oxidative stress monitoring is quantifying their stable end products of oxidative reactions with cellular macromolecules (Orhanl et al., 2004). Dityrosine, as a stable biomarker of ROS mediated protein oxidation and malondialdehyde (MDA) a biomarker of lipid peroxidation are closely correlated with level of oxidative stress (Halliwell and Gutteridge, 1998; Feda et al., 2004). To control the level of ROS and protect the cells they possess a number of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols) and enzymes scavenging ROS, regenerating the active form of the antioxidants and eliminate or reduce the damages caused by them (Alscher et al., 1997). SOD, the first major enzyme found in all aerobes, catalyses the disproportion of super oxide radical to H_2O_2 and dioxygen. The intracellular level of H_2O_2 is regulated by a wide range of enzymes, the most important being catalase and peroxidase (Rusina et al., 2004). Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxified the organic peroxides; it can also act on organic hydroperoxides (Kantol et al., 1988).

Plants growing on Cu-contaminated environments may develop variety of other defense mechanisms against its toxicity. Among plants, Cu-tolerant genotypes are better able to protect them against homeostatic disturbance and cellular damages by evoke the antioxidant enzymes induction as a general response to toxic effects of heavy metals (Van-Assche and Clijsters, 1990). The extent of such tolerance and degree of adaptation is highly variable in which the efficiency and capacity of detoxification mechanisms play an important role (Lombardi and Sebastiani, 2005). Additionally, a network of sequestration activities and immobilization functions regulate the uptake, distribution and detoxification of excess metal ions in plants (Clemens, 2001). In the present work, field surveys have been carried out on the plants growing on copper mine in Kerman state. The aim of this study was to investigate the Cu-accumulating ability of three wild type plants enzyme activities and the levels of oxidative damage products of lipids and proteins to clarify some aspects of the plants toxicity tolerance.

2. Materials and methods

2.1. Description of copper mine area and study site

This study was carried out at Sarcheshmeh, located in Sirjan at Kerman province (Longitude: 55°52′20″ E, Latitude: 29°56′40″ N). The rainfall was around 540 mm and there were no industries nearby. The maximum of temperature was +32 °C and the average annual air temperature was 14 °C. Two zones were considered for plant and soil sampling after a geobotanical survey. The locality of Zone 1 was in center of Cu-mine and Zone 2 was at approximately 11.4 km south of waterlogged area of Cu-mine and the ecological conditions were similar in both areas. The soil of zone 2 had never received sources of Cu. Copper-mine was one of the well-known copper mine where the main activity was copper extraction at 1.12% grade of copper. Tailing have been abandoned for 12 years at the time of sampling.

2.2. Plant and soil sampling

Three plant species; *Datura stramonium*, *Malva sylvestris* and *Chenopodium ambrosioides*, that were the commonest native wild type plant species and endemic, naturally grow up on the studied fields in the mine and vicinity considered for this study. Their growth periods were at the same season in both zones. At each site, plant samples were collected at a determined time of single growing season and according to the actual landform of copper-mine and the distribution of vegetation before flowering period.

Care was taken to collect each plant species samples from both zones while they were at same age of growth period. For each species 5–8 plants were collected randomly within the sampling area. Fresh tissues and mature leaves were used for analysis. Plant species were cleaned in abundant deionised fresh water, rinsed with distilled water and personally identified by expert botanist. Due care was taken to avoid metal contamination in the process of sampling, washing, drying and grinding. Corresponding soil samples were also collected at the location of plant sampling from rooting zone (maximum sampling depth about 30 cm) and transferred to a polythene bags. Excess air was squeezed out, the bags sealed, transferred to the laboratory and stored at 4 °C for maximum of 48 h prior to analysis. These samples were then air-dried and sieved

through a 2 mm plastic screen. There were six replicates for each soil sample.

2.3. Soil analysis

Dried soil samples were digested with $HCl + HNO_3 + HClO_4$ (3:1:1, v/v) (Yuan, 1988). Total Cu and other metals were determined by atomic absorption spectrophotometer (Analyst 100, Perkin Elmer, USA), using an acetylene-air flame. Diethylenetriaminepentaacetic acid (DTPA)-extractable Cu, Cd, Co, Zn and Pb contents of 10 g soil samples (sample: DTPA, 1:2, w/v) were determined by atomic absorption spectrophotometer (Page et al., 1982). The reagents and standards for AAS were ultra pure. The detection limits for total and extractable metals in soils were (in mg/K^{-1}): 0.06 for Cd, 0.15 for Co, 0.17 for Pb, 0.08 for Cu and 0.11 for Zn. This step represents the fraction that is water soluble and most easily available to plants and easily leacheable into the groundwater (Siebe, 1995). Soil nitrate (NO_3^-) was analysed according to the method of Primo and Carrasco (1973). The total Kjeldahl nitrogen (TKN) was determined by the method outlined in Bermen and Mulvaney (1982). The pH and electrolytic conductivity (EC) were determined in a water:soil extract 1:1 using a Beckman pH-meter and a conductivity meter model HI8633, Hanna Instruments Co., respectively.

2.4. Plant biomass and Cu content analysis

The washed plants were separated into roots and shoots, and dried in an oven at 60 °C for 48 h, then biomass (DW) was measured. For elemental analysis, the dried plant tissues were ashed in a muffle furnace at 550 °C for 24 h. The ash was digested with a mixture of HNO_3 and $HClO_4$ [5:3 (v/v)], heated on an oven. After cooling, the extracts were diluted and made up to 25 ml with 1 M HNO_3. Copper concentration of the extract was determined by atomic absorption spectrophotometer.

2.5. Chlorophyll determination

Fresh and mature leaves (0.5 g) were extracted with 10 ml 80% acetone as described by Alan (1994). The absorbance of extract was measured at 663 and 645 nm in the UV–Vis light spectrophotometer (model UV-9100). The chlorophyll content was calculated using the equation as followed: $C_T = 20.2\ A_{645} + 8.02\ A_{663}$.

2.6. Chloroplast isolation

Fresh and mature leaves (5 g) were homogenized for 15 s with a homogenizer in 50 ml ice-cold grinding medium containing: 0.33 M sorbitol, 1 mM EDTA, 0.1% BSA, 2 mM sodium ascorbate and 50 mM K_2HPO_4, pH 7.5. The homogenate was filtrated through Miracloth and centrifuged for 1 min at 1000*g* at 4 °C to remove whole cells and cell debris. The intact chloroplasts were pelleted through centrifugation at 4500*g* for 30 s and were gently resuspended in the same buffer without BSA and centrifuged again at the same conditions. This washing procedure was repeated twice and pelleted chloroplasts were isolated (Rusina et al., 2004).

2.7. Vacuole isolation

Leaves were floated on an enzyme solution containing 1 mM $CaCl_2$, 500 mM sorbitol, 0.05% (w/v polyvinylpyrrolidone), 15 mM MES/Tris pH 5.5, 0.2% (w/v) bovine serum albumin, 1% (w/v) cellulose, 0.5% (w/v) Macerozym, 0.01% (w/v) pectolyase, and agitated for 30 min. Vacuoles were released into the recording chamber by hyposmotic shock treatment of protoplasts in 100 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiotheritol (DTT) and 5 mM Tris/MES, pH 7.5, adjusted to $\pi = 300$ mOsm with D-sorbitol. After setting of the vacuoles, hypotonic solution was carefully replaced by standard bath solution (Scholz-starke et al., 2004).

2.8. Measurement of dityrosine

1.2 grams of fresh tissue material were homogenized with 5 ml of ice-cold 50 mM HEPES-KOH, pH 7.2, containing 10 mM EDTA, 2 mM PMSF, 0.1 mM *p*-chloromercuribenzoic acid, 0.1 mM DL-norleucine and 100 mg polyclar AT. The plan tissue homogenate was centrifuged at 5000*g* for 60 min to remove debris. Purification of *o*,*o*′-dityrosine in the clear tissue homogenized supernatant fluid was accomplished by preparative HPLC.

o,*o*′-Dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250 mm × 10 mm) (Orhanl et al., 2004). The composition of eluent varied linearly from acetonitrile–water–TFA (1:99:0.02) to acetonitrile–water–TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. *o*,*o*′-Dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS 2 (150 mm × 4.6 mm, 5 μm) HPLC column (Bester, Amsterdam, The Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. A standard dityrosine sample was prepared according to Amado et al. (1984). Dityrosine was quantified by assuming that its generation from the reaction of tyrosine with horseradish peroxidase in the presence of H_2O_2 was quantitative (using the extinction coefficient $e_{315} = 4.5\ mM^{-1}\ cm^{-1}$ at pH 7.5).

2.9. Malondialdehyde analysis

Proteins of tissue homogenate were precipitated with 40% trichloracetic acid (TCA), w/v. The MDA assay was

based on the condensation of one molecule malondialdehyde with two molecules of thiobarbituric acid (TBA) in the presence of reduced reagent volumes to increase sensitivity, generating a chromogen with UV absorbance. The TBA + MDA complex was analyzed by HPLC essentially as described by Bird et al. (1983). Briefly, the HPLC system consisted of a Hewlett + Packard 1050 gradient pump (Avondale, PA) equipped with an automatic injector, a 1050 diode-array absorption detector and a personal computer using Chem Station Software from Hewlett + Packard. Aliquots of the TBA + MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μm filter (Millipore, Bedford, MA). The flow rate was 2 ml/min. MDA + TBA standards were prepared using tetraethoxypropane. The absorption spectra of standards and samples were identical with a characteristic peak at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the sample protein content. Protein content was determined by the method of Bradford, with standard curves prepared using BSA (Bradford, 1976).

2.10. Preparation of enzyme extracts

Whole tissue (leaves, stems and/or roots) were homogenized (1:5 w/v) separately in an ice cold mortar using 50 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl, 1% polyvinylpyrrolidone and 1 mM EDTA. After centrifugation (20000*g*, 15 min), the supernatant (crude extract of leaves) was used to determine enzyme activities, which were measured at 25 °C.

Catalase (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction co-efficient 0.0394 mM cm^{-1}) at 240 nm for 30 s (Aeby, 1984). The assay mixture containing 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 μl leaf extract in a 3 ml volume. Unit was defined as μmol H_2O_2 decomposed per 1 min. To detect Glutathione peroxidase [EC 1.11.1.9 (GSH-Px)] activity, the method of Hopkins and Tudhope, with *t*-butyl hydroperoxide as a substrate was used (Hopkins and Tudhope, 1973). The reaction mixture comprised 50 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 0.28 mM NADPH, 0.13 mM GSH, 0.16 U GR, 0.073 mM *t*-butyl hydroperoxide and enzyme extract (50 mg protein). One unit of GSH-Px activity was defined as the amount of enzyme that catalyzed the oxidation of NADPH [mmol min^{-1} mg^{-1} protein]. SOD activity was determined by the method of Minami and Yoshikawa with 50 mM Tris-Ca-codylic sodium salt buffer, pH 8.2, containing 0.1 mM EDTA (Minami and Yoshikawa, 1979). The reaction mixture was composed of 1.42% Triton X-100, 0.055 mM nitroblue tetrazolium (NBT), 16 mM pyrogallol and enzyme extract (50 mg protein). The principle of this reaction is based on the measurement of the concentration of the reduced form of NBT determined at 540 nm. The unit (50% inhibition) was established according to the definition of McCord and Fridovich (1969). Unit was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% per 1 min.

2.11. Statistical analysis

All statistical analysis was carried out by using procedure available in the SPSS v.10 (SPSS INC., Chicago, IL) statistical package. Each experiment ($n \geqslant 3$) was run at least in duplicate and the data presented are given as mean ± SD. Student's *t*-test was applied to determine the significance of results between different samples. Statistical significance was set at the $P < 0.05$ confidence level.

3. Results

Table 1 shows the chemical characteristics of the soil samples that were collected at the locations of plant sampling. Evaluations of EC level and pH parameter revealed that the water extracts of the soils in both zones were mild acidic, however the soil samples associated with plants in zone 1 had slightly lower pH than zone 2. In addition, there was no problem with salinity. To better characterized nitrogen species levels, we measured total nitrogen and nitrate levels, and the results indicated that both parameters were slightly lower in zone 1 than zone 2, although they did not differ significantly. Total contents of each metal; Cd, Co, Zn and Pb in the soil samples of zone 1 were significantly higher than those of zone 2 soils. On the other hand, the levels of these metals were generally below the maximum allowable concentration of the USA (Kabata-Pendias, 1995).

The ratio of total Cu in zone 1 to that in zone 2 was around 50 folds and about 85 folds for available Cu, the levels that were higher than toxicity threshold levels (ICRCL, 1987). However, the available Cu concentration for plants in zone 1 consisted 37% of total cu level. There were no significant differences in the available levels of the studied metals between soils of the zones except for Cu. The soils of zone 2 displayed no exceptional high metal concentrations, the levels that could not be toxic for plants; particularly cobalt and cadmium were normally low. Table 2 compares the contents of Cu in roots, leaves and stems of three plant species collected from different sites. The Cu levels in stems, leaves and roots varied with plant species. In general, all species in zone 1 contained significantly higher Cu concentrations in their tissues than those growing on zone 2 soils, except *M. sylvestris*. In *M. sylvestris* species growing on zone 1, the Cu level of stems and/or roots were insignificantly lower than those of zone 2. Moreover, and apart from the parameter of living zone, as we compared the levels of Cu between tissues in *C. ambrosioides* and *M. sylvestris*, we found significant increase in copper level in roots, stems and leaves respectively. The roots of *C. ambrosioides* accumulated Cu up to about five folds of its level in leaves and to

Table 1
Chemical characteristics of soils of the studied zones[a]

Zone	Heavy metal content [mg/(kg dw)]										pH	$E \cdot C$ (mS/cm)[c]	Nitrogen (g/kg dw)	
	Cu		Cd		Co		Zn		Pb					
	T	E	T	E	T	E	T	E	T	E			NO_3^-	Total
1	924.5 ± [b]41.6	341.6 ± [b]17.1	3.15 ± 0.82	<0.19	9.2 ± 2.64	<0.10	44.86 ± [b]8.2	3.15 ± 0.47	13.6 ± [b]3.81	<0.25	5.73 ± 0.46	2.11 ± 0.21	0.22 ± 0.03	1.47 ± 0.10
2	18.41 ± 3.65	4.02 ± 0.81	1.88 ± 0.67	<0.13	3.30 ± 1.73	<0.17	29.72 ± 5.8	4.16 ± 0.54	6.7 ± 1.74	<0.25	6.58 ± 0.29	1.82 ± 0.14	0.28 ± 0.02	1.61 ± 0.14

[a] T, total content; E, DTPA-extractable content. Data were presented as mean ± SD.
[b] Significant difference with respect to zone 2 ($P < 0.05$).
[c] Electrolytic conductivity in water:soil extract (1:1).

two folds of its content in stems. On the contrary, *D. stramonium* showed significantly high Cu contents in leaf, stem and roots, respectively. The level of Cu in leaves of *D. stramonium* rose up to around three folds of its total content in roots.

The roots of *C. ambrosioides* grown on zone 1 accumulated most of Cu as compared with the tissues of other plant species. On the other hand, the copper levels in shoots (leaves + stems) of *C. ambrosioides* species and in leaves of *D. stramonium*, were above the critical level for copper toxicity (Robson and Reuter, 1981). The ratio of Cu in roots of *C. ambrosioides* and/or *D. stramonium* growing on zone 1 to that in the roots of the same plants in zone 2 were approximately 9 and 7, respectively. The level of Cu in leaf tissues vacuoles and chloroplasts and their ratios to total leaves copper (as percentage) are presented in Table 3. In general, all species in zone 1 had significantly higher Cu content in their vacuoles and/or chloroplasts with respect to the same plant species in zone 2. The concentration of copper in vacuoles of *D. stramonium*, *C. ambrosioides* and *M. sylvestris* were 27.21, 7.92 and 0.54, respectively. Most of Cu was accumulated in vacuoles of *D. stramonium* leaves, consisted 42% of total leaves Cu levels and was over five times higher than its chloroplast Cu content. Among three species, the level of copper in chloroplasts of *C. ambrosioides* leaves was significantly higher than other species chloroplasts and also two times higher than its vacuoles Cu level. *M. sylvestris* growing on zone 1 showed the lowest vacuoles and chloroplasts copper levels among three plant species.

The biomass characteristic of plant species and their leaves chlorophyll contents are exhibited in Table 4. In *C. ambrosioides* associated with zone 1, the biomass of shoots (as above ground part of plant) increased significantly with respect to those of zone 2, although, there was insignificant increase in dry weight of its roots. The leaves chlorophyll contents of this plant species and *M. sylvestris* in zone 1 were also significantly higher than those of zone 2. On the other hand, the increase in shoot and decrease in roots biomass levels of *M. sylvestris*, collected from zone 1 were insignificant as compared with zone 2. The biomass and chlorophyll contents of *D. stramonium* from zone 1 were insignificantly lower than the same plant species of zone 2.

Table 5 demonstrated antioxidative enzyme activities of different parts of the studied plants. In *C. ambrosioides* and *M. sylvestris*, the enzyme activities were higher in leaves, stems and roots, respectively in both zones. As comparison, only enzyme activities in tissues of *C. ambrosioides* and *D. stramonium* were significantly higher than those of zone 2. In zone 1, each tissue of *C. ambrosioides* revealed significant increase in SOD and CAT activities with respect to *M. sylvestri*. In addition, the leaves of both species had significantly higher SOD and CAT activities than roots. On the other hand, there was only significant increase in CAT activity in stems of *C. ambrosioides* as compared with roots.

Table 2
Copper bioconcentration (g/kg dw) in tissues of studied plants[a]

Plant	Zone 1			Zone 2		
	Leaf	Stem	Root	Leaf	Stem	Root
Malva sylvestris	7.82 ± 1.36^{b}	9.44 ± 1.91^{b}	21.35 ± 2.68	$5.54 \pm 1.17^{c,b}$	11.63 ± 1.83	28.17 ± 4.53
Chenopodium ambrosioides	$88.1 \pm 10.3^{c,b}$	192.5 ± 18.6^{b}	417.6 ± 31.1	12.1 ± 1.6^{b}	16.2 ± 2.3	43.6 ± 5.1
Datura stramonium	$64.61 \pm 5.80^{c,b}$	31.54 ± 3.12^{b}	19.16 ± 2.31	$6.62 \pm 1.26^{c,b}$	3.81 ± 0.77	2.74 ± 0.52

[a] Data were presented as mean ± SD.
[b] Statistically differ with respect to the roots.
[c] Statistically differ with respect to the stems.

Table 3
Copper bioconcentration (μg/g dw) in the leaf vacuoles and chloroplasts of studied plants[*]

Plant	Zone 1				Zone 2			
	Chloroplasts		Vacuoles		Chloroplasts		Vacuoles	
	Cu level (μg/g dw)	%[a]	Cu level (μg/g dw)	%[a]	Cu level (μg/g dw)	%[a]	Cu level (μg/g dw)	%[a]
Malva sylvestris	0.86 ± 0.08^{b}	11	0.54 ± 0.05	7	0.44 ± 0.04	8	0.27 ± 0.01	5
Chenopodium ambrosioides	15.85 ± 1.35^{b}	18	7.92 ± 0.81	9	1.82 ± 0.24	15	1.7 ± 0.19	14
Datura stramonium	5.18 ± 0.76^{b}	8	27.21 ± 2.62	42	0.26 ± 0.01	4	1.12 ± 0.16	17

[*] Data were presented as mean ± SD.
[a] The rate of leaf vacuoles Cu to total leaf tissue Cu (as percent).
[b] Significant difference with respect to vacuoles ($p < 0.05$).

On the other hand, the increases in these enzyme activities of *D. stramonium* growing on zone 1 were considerable for roots, stems and then leaves respectively, but in zone 2, the increase pattern were observed in leaves, stems and roots, respectively.

Moreover, *D. stramonium* showed significant increase in SOD and CAT activities of roots with respect to stems and/or leaves in zone 1. GPX activity of *D. stramonium* increased significantly only in roots with respect to leaves. There were also insignificant increases in antioxidative enzymes parameters of stems, roots and leaves of *M. sylvestris* associated with zone 1 as compared with zone 2. In *M. sylvestris*, among these enzymes, only CAT activity of leaves was significantly higher than roots in both zones. However in zone 1, there was only significant increase in SOD activity of *M. sylvestris* leaves with respect to roots.

In this plant, there were no significant differences in the activities of SOD and/or GPX of stems as compared with roots or leaves. The levels of oxidative damage biomarkers of lipids as MDA and proteins as dityrosine are shown in Table 6. Both parameters were insignificantly higher in tissues of *C. ambrosioides* and *M. sylvestris* species that were grown on zone 1 as compared to the same plant species in zone 2. In these two species, the levels of these biomarkers were higher in roots, stems and leaves, respectively in both zones. There were significant differences between roots and leaves for these parameters levels. On the contrary, In *D. stramonium*, the levels of oxidative damages were considerable in leaves, stems and roots, respectively in zone 1, but in zone 2, the increase pattern was remarkable for roots, stems and leaves, respectively. The studied parameters in leaves of this plant species growing on zone 1 were significantly higher in comparison with the same plants of zone 2.

4. Discussion

In this work, the contaminated field of study was located in copper mine. Accordingly, the soil analysis revealed normal levels of heavy metals (Pb, Co and Cd) and toxic level of Cu in which the rate of available concentration of this metal was quit high (around 37%) for plants growth. This high Cu availability may be attribute to our soil pH characteristic. It has been confirmed that low level of this parameter cause the increase in Cu solubility and its release from the soil phase leading to the elevation in copper uptake by roots (Watmough and Dickinson, 1995). Our results were in agreement with the findings, reported in Cyprus Skouriotissa Cu mine, where pH was mild acidic and contained copper up to 787 mg $(kg\ DW)^{-1}$ (Johansson et al., 2005). In our zones of investigation, normal growth of our studied plants in metalliferous soils without any visual and conspicuous symptoms of Cu toxicity necessarily implied that they were tolerant to toxic levels of Cu. These plant species were endemic in Cu mine and have adapted to contaminated soils by developing tolerance mechanisms to metal stress. Most of these mechanisms have been recognized prior as exclusion, accumulation of metals and internal protective responses that vary among plant species and among different tissues (Freitas et al., 2004; Nicolau et al., 2005).

Our study on tissues of *M. sylvestris*, grown in Cu-contaminated zone, revealed low level of Cu in stems

Table 4
Chlorophyll content and biomass of plant tissues[a]

Plant	Zone 1			Zone 2		
	Biomass [mg/(g FW)]		Chlorophyll [mg/(g FW)]	Biomass [mg/(g FW)]		Chlorophyll [mg/(g FW)]
	Shoot (Stem + leaf)	Root		Shoot (Stem + leaf)	Root	
Malva sylvestris	53.39 ± 4.41	36.52 ± 3.61	2.11 ± 0.29	48.26 ± 3.89	43.17 ± 4.22	1.59 ± 0.23
Chenopodium ambrosioides	73.52 ± [b]5.24	49.11 ± 3.75	1.06 ± [b]0.12	62.15 ± 4.32	43.22 ± 3.51	0.71 ± 0.08
Datura stramonium	86.74 ± 5.16	70.37 ± 4.71	1.27 ± 0.18	91.33 ± 5.42	78.40 ± 4.52	1.48 ± 0.25

[a] Data were presented as mean ± SD.
[b] Significant difference with respect to zone 2 ($P < 0.05$).

and/or roots as compared with those of zone 2. In agreement with our findings, the investigation on *Rumex dentatus* plant grown on soil with high level of copper, showed insignificant difference in Cu contents of roots as compared with the samples collected from control condition (Jie et al., 2004). On this basis, we can conclude that *M. sylvestris*, possess more ability to exclude Cu from up taking and transporting with respect to other studied plants. Our conclusion is in accordance with this finding illustrated that metal exclusion and/or metal ion stabilization in the soil by mechanisms in the roots, can restrict copper bioavailability and its uptake, leading to retardation of Cu accumulation (Salt et al., 1995) and protection against phytotoxicity of copper. As another indication for metal tolerance, McGrath et al. (2001) demonstrated that metal exclusion is more common strategy than metal accumulation. On the other hand, *M. sylvestris* in zone 1 revealed insignificant decrease in roots biomass as compared with zone 2. This decrease in growth parameter could be the consequence of additional energy cost in relation with metal exclusion mechanism of root tissue (Lefebvre and Vernet, 1990). We hypothesized that the metal exclusion mechanism was promoted by high concentration of Cu in contaminated zone, that leading to decrease in Cu content in shoots of *M. sylvestris* with respect to the same plant in zone 2. In addition, different investigations reported decrease in chlorophyll content of plants after exposure to high level of copper (Devi and Prasad, 1998; Prasad et al., 2001). They attributed chlorophyll decrease to Cu modification of chlorophyll degradation and its structural and functional damage (Prasad et al., 2001). Accordingly, we concluded that slightly lower content of Cu in leaves of *M. sylvestris* in zone 1 caused lower damage to chlorophyll structure, resulted more chlorophyll level with respect to zone 2. Another consequence of conservation of this metal at low level this plant tissues in zone 1 was the lack of considerable induction in antioxidative enzyme activities in this species with respect to control condition and to other studied plants. In spit of *M. sylvestris*, two other species; *D. stramonium* and *C. ambrosioides* both, had the ability to accumulate copper in their tissues. However, because their Cu contents were lower than 1000 μg/g, a threshold limit that is prescribed for hyperaccumulators (Reeves and Baker, 2000), they could not be considered as hyperaccumulators. As comparison, we found higher Cu accumulation in *C. ambrosioides* with respect to *D. stramonium*. This finding was in agreement with the reports of Shu et al. (2002) and Brun et al. (2001), illustrated that metal accumulation ability vary between species and is affected by their intrinsic characteristics. In their study, Cu was accumulated differently in *Paspalum distichum* and *Cynodon dactylon*, as metal tolerant plants, collected from Lechang tailing copper mine from China. They also found higher copper contents in roots of *Cynodon dactylon* and in shoots of *Paspalum distichum* with respect to their other tissues. Such patterns of copper bioaccumulation and partitioning among different parts of tolerant plants have been

Table 5
Antioxidant enzyme activities in different plant tissues[a]

Plant	Enzyme	Zone 1			Zone 2		
		Leaf	Stem	Root	Leaf	Stem	Root
Malva sylvestris	SOD (U/mg protein)	8.71[c] ± 1.91	6.38 ± 2.18	5.73 ± 1.93	6.11 ± 1.85	3.62 ± 1.14	4.21 ± 1.38
	CAT (μmol/min/mg)	29.34[c] ± 3.73	23.12 ± 3.21	20.19 ± 3.45	23.11[c] ± 3.17	17.66 ± 2.81	14.51 ± 2.52
	GPX (U/mg protein)	4.79 ± 0.93	3.16 ± 0.88	3.91 ± 0.82	3.61 ± 0.84	1.94 ± 0.38	2.39 ± 0.75
Chenopodium ambrosioides	SOD (U/mg protein)	48.35[c] ± [b]4.61	31.62 ± [b]3.67	25.63 ± [b]3.25	19.34 ± 3.13	10.82 ± 1.94	14.51 ± 2.56
	CAT (μmol/min/mg)	94.74[c] ± [b]6.51	65.63[c] ± [b]5.83	50.34 ± [b]5.35	34.25[c] ± 3.62	27.53 ± 2.83	22.14 ± 2.14
	GPX (U/mg protein)	8.15 ± [b]1.87	6.32 ± [b]1.14	5.12 ± [b]1.22	2.17 ± 0.52	1.43 ± 0.38	1.15 ± 0.31
Datura stramonium	SOD (U/mg protein)	25.21[c] ± [b]2.84	28.64[c] ± [b]3.02	35.19 ± [b]3.23	14.23[c] ± 2.57	9.83 ± 2.31	7.51 ± 1.73
	CAT (μmol/min/mg)	52.61[c] ± [b]5.14	60.35 ± [b]5.73	81.47 ± [b]6.32	28.44 ± 2.61	35.36 ± 3.43	18.42 ± 2.21
	GPX (U/mg protein)	5.22[c] ± [b]1.26	7.73 ± [b]1.74	10.82 ± [b]2.27	5.71 ± 1.34	4.14 ± 1.15	2.80 ± 0.86

[a] Data were presented as mean ± SD.
[b] Significant difference with respect to zone 2 ($p < 0.05$).
[c] Statistically differ with respect to the roots.

reported in many studies (Marschner, 1995; Mulligan et al., 2001; Pyatt, 2001; Stoltz and Greger, 2002). As another indication, *Pistacia terebinthus* and *Cistus creticus* collected from Skouriotissa Cu mine, accumulated considerable amount of the absorbed Cu in their roots, although *Bosea cypria* accumulated most of Cu in leaves (Johansson et al., 2005). In agreement with these documents our studied plants, *D. stramonium* and *C. ambrosioides*, both showed Cu accumulation partitioning. In *C. ambrosioides*, most of Cu was accumulated or bounded in roots with restriction in translocation of copper toward shoots. On the other hand, *D. stramonium* was able to absorb, transport and store the copper into the above ground parts, particularly into the leaves.

It has been confirmed in many studies that when copper is in excess, it can promote and stimulate generation of Fenton-type reactive oxygen species leading to increase in antioxidant enzyme activities as a defense system (Weckx and Clijesters, 1996; Devi and Prasad, 1998; Lombardi and Sebastiani, 2005). This response to excess copper can vary among plant species and among different tissue (Lombardi and Sebastiani, 2005). Accordingly, the observed increase in each antioxidative enzyme activity in *C. ambrosioides* from Cu-contaminated zone could be due to the induction of excess Cu. We also conclude that the induction levels on the studied enzyme activities in *C. ambrosioides* were sufficient to protect proteins, chlorophyll and lipids of some parts of plants against ROS attack. On this basis, the biomass of each plant part and leaves chlorophyll content of this plant associated with zone 1 were insignificantly higher than the same plant growing on zone 2, although they did not differ significantly in MDA and dityrosine levels. As comparison, the roots of *C. ambrosioides* revealed significant increase in MDA and dityrosine with respect to leaves that may be attribute to considerable low activities of antioxidative enzymes in roots. Due to our documents, many studies illustrated inhibition effect on antioxidative enzymes by excess copper (Luna et al., 1994; Maribel and Satoshi, 1998). Because of higher copper content in the roots of *C. ambrosioides*, it would be exerted toxic effect on antioxidant enzymes leading to significant decrease in their activities with respect to leaves. On the basis of our findings and illustrated documents, it is our conviction that antioxidative enzymes play a key role in defense system against oxidative damages and in tolerance of *C. ambrosioides* in Cu-rich environment. Apart from these mechanisms, the immobilization of excess heavy metals via their storage in cell walls (Hughes and Williams, 1988) or accumulation in vacuoles (McCain and Markley, 1989), were suggested as another strategy to increase the plant internal tolerance against Cu toxicity. With regard to these documents, we found that *D. stramonium* from zone 1 accumulated around 42% of total Cu in the leaves vacuoles that was markedly higher as compared with two other plant species. This organelle Cu-accumulation caused the decrease in the levels of copper out of vacuoles, leading to low induction on antioxidative enzymes in the leaves as compared with roots of *D. stramonium*. Accordingly, significant increase in MDA and dityrosine in leaves with respect to roots of *D. stramonium* could be the consequence of considerable low activities of antioxidative enzymes in leaves. However, slightly loss in chlorophyll content may be due to lipid peroxidation of chloroplast and thylakoid membranes and chlorophyll degradation mediated by Cu (Baszynski et al., 1988; Vinit-Dunand et al., 2002). In accordance to our finding, growing of *Cladonia convoluta* on Cu mine with copper content exceeding 175 $\mu g\ g^{-1}$ dry weight caused a decrease in total chlorophyll level (Chettri et al., 1998).

Moreover, although *D. stramonium* leaves in zone 1 showed significant increase in oxidative damage parameters with respect to zone 2, the parameter of biomass decreased insignificantly in this tissue of plant at zone 1. We suppose that the observed activity of antioxidative enzymes was insufficient for full protection of *D. stramonium* tissues particularly its leaves against ROS damages. Accordingly, further investigations on tolerance mechanisms of *D. stramonium* are needed. It is our conviction that our studied

Table 6
The levels of lipid peroxidation and protein oxidation biomarkers in tissues of studied plants[a]

Plant	Zone 1						Zone 2					
	MDA (nmol/mg protein)			Dityrosine (nmol/mg protein)			MDA (nmol/mg protein)			Dityrosine (nmol/mg protein)		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Malva sylvestris	13.39 ± 2.21	14.18 ± 2.63	21.36 ± [b]3.25	0.86 ± 0.19	1.09 ± 0.23	1.38 ± [b]0.32	9.75 ± 1.66	12.28 ± 2.20	15.49 ± [b]2.78	0.72 ± 0.18	0.89 ± 0.21	1.07 ± 0.26
Chenopodium ambrosioides	10.15 ± 2.77	16.34 ± 3.09	18.22 ± [b]3.28	1.07 ± 0.26	1.59 ± 0.28	1.95 ± [b]0.38	7.50 ± 1.74	12.17 ± 2.48	14.75 ± [b]2.86	0.98 ± 0.19	1.27 ± 0.24	1.54 ± [b]0.29
Datura stramonium	33.40 ± 3.83	27.26 ± 3.34	24.33 ± [b]2.62	3.27 ± 0.43	2.83 ± 0.24	2.12 ± [b]0.27	18.45 ± 2.16	21.19 ± 2.41	26.33 ± [b]3.12	1.86 ± 0.18	2.17 ± 0.27	2.62 ± [b]0.32

[a] Data were presented as mean ± SD.
[b] Statistically differ with respect to the leaf.

plant species had different degree of tolerance, a characteristic that has been recognized in different plant species growing on copper-rich soil including California copper mine (Kruckeberg and Wu, 1992) and Sao Domingos mine (Freitas et al., 2004). As conclusion, this study showed different tolerance strategies in plant species. In *M. sylvestris*, exclusion of Cu from the roots or its stabilization in the soil restricted Cu toxicity effects. But, antioxidative enzymes responses to Cu-stress, activated in *D. stramonium* and *C. ambrosioides* in addition with accumulation of Cu in leaves vacuoles of *D. stramonium* protected them against oxidative damages and involved in their tolerance in copper mine.

Acknowledgments

This work was supported in part by a conjoint grant (No. 4.1292) from research Ministry of Tarbiat Moallem University. The excellent technical assistance of Faranak Goodarzi, is gratefully acknowledged.

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