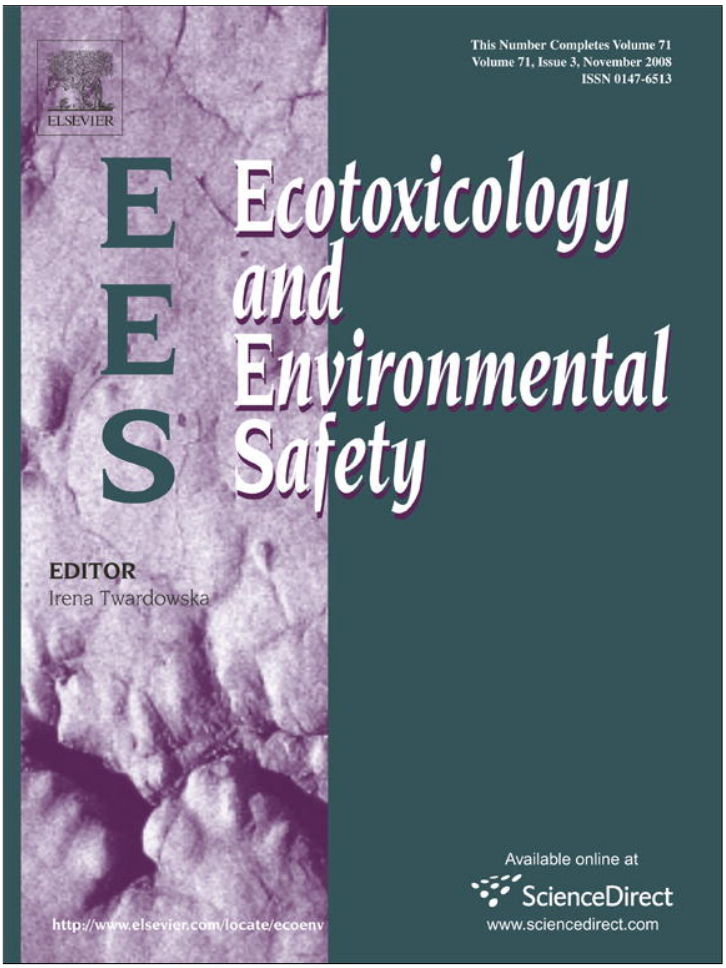

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Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Comparative evaluation of oxidative stress status and manganese availability in plants growing on manganese mine

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Received 29 April 2006; received in revised form 19 September 2007; accepted 21 October 2007
Available online 18 December 2007

Abstract

This study pioneered an approach that determined the effects of excess manganese (Mn) on three species; *Datura stramonium*, *Alhagi camelthorn* and *Chenopodium ambrosioides*. We investigated their levels of Mn, antioxidative enzymes and oxidative damage biomarkers in plants (zone 1) in and outside (zone 2) the Mn mine. The results showed that total and available Mn were at toxic levels for plants growing on zone 1. The Mn levels in each plant species were higher in leaves, stems and roots. Mn was only accumulated significantly in leaf vacuoles of *A. camelthorn*. Antioxidative enzyme activities of *C. ambrosioides* and/or *D. stramonium* in zone 1 were higher in leaves, stems and then in their roots. Malondialdehyde (MDA) and dityrosine levels were insignificantly higher in tissues of the studied plants in zone 1 with respect to zone 2. The roots of studied plants showed significantly higher levels of these biomarkers in comparison with their leaves in zone 1. Accordingly, antioxidative enzymatic response to Mn-stress in *D. stramonium* and *C. ambrosioides* and possibly accumulation of Mn in leaf vacuoles of *A. camelthorn*, protected them from oxidative damages and involved in their tolerance in Mn mine.

Keywords: Manganese mine; Antioxidative enzyme; Malondialdehyde; Dityrosine; Vacuoles; Plant tolerance: Mn accumulation

1. Introduction

Manganese (Mn) as an essential trace element and an activator of a number of enzymes is involved in photosynthesis, respiration and the synthesis of proteins, acyl lipids and carbohydrates in plant systems (Mukhopadhyay and Sharma, 1991). Absorption of Mn by plant organs depends on the ability of the plants to transfer the metal across the soil–root interface and on the total and available amount of Mn in soil (Farasova and Beinrohr, 1998).

At subcellular level, excess Mn might be stored in the vacuoles, cell walls and in chloroplast thylakoids (Gonzales and Lynch, 1999). This metal when present at abnormally high levels in soil becomes cytotoxic and can cause injury and create symptoms in plants that have been associated with brown spots on mature leaves, interveinal chlorosis, early necrotic flecking on stems and growth retardation (Wissemeier and Horst, 1992; Sarkar et al., 2004).

Accordingly, Mn toxicity has important implication for the effects of global change on ecosystems prone to Mn stress in which the role of oxidative stress and reactive oxygen species (ROS) production may be involved (Krupa and Baszynski, 1995). Under normal conditions, production and scavenging of these activated oxygen species are regulated well. However, under metal toxicity, the formation of ROS might be in excess, leading to a disturbance of metabolic pathway and macromolecule damages (Hegedus et al., 2001). ROS can provoke oxidation and modification of cellular proteins, amino acids, membrane lipids and even DNA, creating oxidative injury that results in a reduction

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[1]I hereby certify that all procedures followed in our study were in accordance with those of the responsible committee on human experimentation (Institutional) and with the Helsinki Declaration of 1975, as revised in 1983. This work was conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. In addition, this investigation was supported by grant of Research Deputy of Tarbiat Moalem University.

0147-6513/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2007.10.011

of plant growth and development (Halliwell and Gutteridge, 1998a; Ogawa and Iwabuchi, 2001).

Since the half-lives of ROS are extremely short, their stable end products of oxidative damages to cellular macromolecules can be used for oxidative stress monitoring (Orhanl et al., 2004). Dityrosine, a hallmark of oxidized proteins and malondialdehyde (MDA), a biomarker of lipid peroxidation, are closely correlated with level of oxidative stress (Halliwell and Gutteridge, 1998b).

To control the level of ROS and to protect the cells, plants 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). Antioxidant enzyme defense layer is largely provided by specific enzymes including superoxide dismutase (SOD) as a family of metalloenzymes, catalyses dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen (Alscher et al., 2002). Catalase inactivates the bulk of hydrogen peroxide to oxygen and water. Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxifies the organic peroxide (Mittler, 2002).

Among higher plants, Mn-tolerant genotypes are better able to protect themselves against homeostatic disturbance and cellular damages by evoking the antioxidant enzymes induction as a general response to toxic effects of heavy metals in soil (Van and Clijsters, 1990). Additionally, immobilization of excess Mn in oxalate crystals has been suggested as another detoxification and tolerance mechanism (Gonzales and Lynch, 1999). 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 (Nelson, 1983).

Soils and water with high level of Mn are becoming an environmental concern in relation to the Mn mine of Iran following the increased awareness of the need for environmental protection. For the present work, field surveys have been carried out on the plants growing on the Mn mine in Qum state. The aim of this study was to investigate the Mn-accumulating ability of three wild-type plants *Datura stramonium*, *Alhagi camelthorn* and *Chenopodium ambrosioides* growing wild on the mine. We also evaluated the corresponding status of the antioxidant 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 the manganese mine area and the study site

The Mn mine is located at Qum city, Venarch province, Iran (Latitude 34°20′–34°28′N, Longitude 50°35′–50°55′E), which has tropical continental climate with an annual average temperature 19.4 °C and there are no industries nearby. Two zones were considered for plant and soil sampling. The locality of zone 1 was in center of the Mn mine and zone 2 was at about 3.4 km away from vicinity of Mn mine. The ecological conditions were similar in these areas. Oxide and silicate were the main chemical forms of Mn, which presented as Braunite, the main ore mineral of Mn in the entire plant area.

2.2. Plant and soil sampling

At each zone, plant samples were collected at a determined time of the single growing season, according to the actual landform of Mn mine and the distribution of vegetation at same times and age before flowering period. Three plant species, *D. stramonium*, *A. camelthorn* and *C. ambrosioides*, that were the commonest native wild-type plant species and endemic, naturally grow up on the studied fields in the mine and vicinity were considered for this study. Expert botanist identified the plant species. For each species 5–8 plants were collected within the sampling region and their fresh tissues, including roots, stems and mature leaves, were used for analysis. We had three random regions for sampling at each zone of the study. Each collected plant was considered for three replicate analysis. The tissues, particularly roots were cleaned in abundant deionized fresh water and rinsed with distilled water. Due care was taken to avoid metal contamination in the process of sampling, washing, drying and grinding. At the locations where plants were sampled soil samples were also collected (maximum sampling depth about 30 cm). These samples were then air-dried and sieved through a 2 mm plastic screen.

2.3. Soil analysis

A hydrometric method was used to analyze soil particle size (Day, 1965). Water-holding capacity (WHC) at atmospheric pressure of 1/3 bar was determined and considered as the amount of water held in soil in the upper ring 24 h after drainage of water (Wang, 1989). Cation-exchange capacity was determined by the standard method (Bower et al., 1952).

Dried soil samples were digested with $HCl + HNO_3 + HClO_4$ (3:1:1, v/v) (Yuan, 1988). Mn and other metals were determined by atomic absorption spectrometry (Analyst 100, Perkin-Elmer, USA). The metals in soils were sequentially extracted following a slight modification of the method described by Tessier et al. (1979). The modification consisted of initially extracting with double-distilled water (2 g of soil shaken for 4 h in distilled water of electric conductivity (EC) $<0.02\,\mu S\,cm^{-1}$, followed by centrifugation for 10 min at 3000 rpm). 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 analyzed 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. Measurement of *O,O′*_Dityrosine

The plan tissue homogenate was centrifuged at 5000*g* for 60 min to remove debris. *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 and a standard of dityrosine sample was prepared according to the method of Amado et al. (1984).

2.5. Malondialdehyde analysis

Proteins of tissue homogenate were precipitated with 40% trichloracetic acid (TCA), w/v. Condensation of one molecule MDA with two molecules of thiobarbituric acid (TBA) generated chromogen with UV absorbance. The TBA + MDA complex was analyzed by HPLC essentially as described by Bird et al. (1983). Briefly, the HPLC system used was the one 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 ($30 \times 4.6\,mm^2$). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μm filter (Millipore, Bedford, MA, USA) and the flow rate was 2 ml/min. Measurements were expressed in terms of MDA normalized to the sample protein content.

2.6. 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 (20,000*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 coefficient $0.0394\,mM\,cm^{-1}$) at 240 nm for 30 s (Aeby, 1984). The assay mixture contained 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 nmol 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 (1973), with *t*-butyl hydroperoxide as a substrate, was used. 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 (1979) with 50 mM Tris–Ca–codylic sodium salt buffer, pH 8.2, containing 0.1 mM EDTA. 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 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.

2.7. Determination of metals in plant leaves

Leaves were dried at 60 °C. Dry plant material (0.1 g) was separately ashed at 550 °C and the residue was brought to standard volume with 20% HCl. Each of metal content was determined directly by atomic absorption spectrometry (SP 191 "Unicam"). Protein content was determined by the method of Bradford (1976), with standard curves prepared using BSA.

2.8. Vacuole isolation

Leaves were let float 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 from protoplasts in standard solution that 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).

2.9. Statistical analysis

All statistical analysis was carried out by using the procedure available in the SPSS v.10 (SPSS Inc., Chicago, IL, USA) statistical package. Each experiment was run on each sample at least three times, then we calculated mean ± SD of test results obtained from all samples collected from all parts for each zone and the data are presented 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. When testing for relationships, the sample parameters at each zone were considered separately and Pearson's correlation coefficients were calculated.

3. Results

The chemical characteristics of soils are shown in Table 1. The pH of the water extracts of soils, EC, cation exchange capacity (CEC) and WHC did not differ significantly between zone 1 and zone 2. The pH levels indicated that both zones were mild acidic, in addition there was slightly higher clay content in soil samples of zone 1. To better characterize nitrogen species levels, we measured total nitrogen and nitrate. The results showed that these parameters were slightly lower in zone 1 than in zone 2, although they did not differ significantly. The total levels of Cd and/or Hg were generally below the maximum allowable concentration of the European Union countries and USA, except for total and available Mn (Kabata-Pendias, 1995).

Concentrations of Mn in different parts of the studied plants are illustrated in Table 2. In three plant species of zone 1, the concentrations of Mn were higher in leaves than in stems with respect to roots in which there were

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

Zone	Total contents (mg/kg dw)			AC (mg/kg dw)	EC (ds/m)[b]	Clay (g/kg)	CEC (cmol/kg)	WHC (m^3/m^3)	pH (H_2o)	NO_3^- ($g\,kg^{-1}$ dw)	Total nitrogen (g/kg dw)
	Cd	Hg	Mn	Mn							
1	0.47 ± 0.06	< 0.01	127,215[c] ± 7324	78,186[c] ± 4115	1.11 ± 0.09	19.6 ± 4.6	291 ± 37	0.27 ± 0.04	6.24 ± 0.23	0.17 ± 0.01	1.47 ± 0.10
2	0.38 ± 0.05	< 0.01	11,062 ± 1213	6140 ± 872	1.06 ± 0.07	12.1 ± 3.8	228 ± 26	0.36 ± 0.05	6.58 ± 0.27	0.19 ± 0.01	1.61 ± 0.14

AC—available concentration; CEC—cation exchange capacity; WHC—water-holding capacity.
[a]Data were presented as mean ± SD.
[b]Electrolytic conductivity in water:soil extract (1:1).
[c]Significant difference with respect to zone 2 ($p < 0.05$).

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

Plant	Zone 1			Zone 2		
	Leaf	Stem	Root	Leaf	Stem	Root
Datura stramonium	2468±108[b,c]	1174±68[c]	947±48	324±29[b,c]	252±24	226±26
Alhagi camelthorn	2035±96[c]	1846±93[c]	872±51	386±34[c]	343±31	316±28
Chenopodium ambrosioides	3251±134[b,c]	1411±87[c]	1127±62	598±38[b,c]	417±34	354±30

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

Table 3
Manganese bioconcentration (μg/g dw) in the vacuoles of leaf tissues of studied plants[a]

Plant	Zone 1		Zone 2	
	Leaf vacuoles	%[b]	Leaf vacuoles	%[b]
Datura stramonium	637±32.4[c]	26	46±5.9	14
Alhagi camelthorn	862±40.5[c]	42	84±6.8	21
Chenopodium ambrosioides	512±26.8[c]	16	61±5.2	11

[a]Data were presented as mean±SD.
[b]The rate of leaf vacuoles Mn to total leaf tissue Mn (as percent).
[c]Significant difference with respect to zone 2 ($p<0.05$).

significant differences between aerial parts and roots of *D. stramonium* and *C. ambrosioides*.

In *A. camelthorn*, the concentration of Mn in the leaves was insignificantly higher than stems and significantly greater than roots in zone 1. In zone 2, statistically significant increase in Mn levels were seen in leaves as compared to roots in all species. However, the stems and roots did not differ significantly in Mn level. The ratio of Mn in the leaves of *D. stramonium* and/or *C. ambrosioides* in zone 1 to that in the leaves of the same plants in zone 2 were approximately 7 and 5, respectively.

The level of Mn in leaf vacuoles and its ratio to total leaf Mn (as percentage) is presented in Table 3. These percentages were 42% and 26% for *A. camelthorn* and *D. stramonium* and the percentage was 16% for *C. ambrosioides*. The accumulated percentage of Mn in leaf vacuoles of *A. camelthorn* was around two fold of that of *D. stramonium* and three fold of that of *C. ambrosioides*. Statistically these differences were significant.

Antioxidative enzyme activities of different parts of the studied plants are presented in Table 4. In *C. ambrosioides* and/or *D. stramonium*, antioxidative enzyme activities were higher in leaves, stems and roots, in both zones.

In zone 1, the aerial parts of *C. ambrosioides* and of *D. stramonium* showed high levels in SOD and CAT activities with respect to their roots, in which the differences were significant when leaves were compared with roots and/or stems.

These enzyme activities in leaves, stems and roots of *D. stramonium* and/or *C. ambrosioides* in zone 1 were significantly grater than those in samples of the same plants in zone 2. In *A. camelthorn*, among these three enzymes, only CAT activity of leaves was significantly higher than roots in both zones, but for SOD, this significant increase in leaves was observed only in zone 1. In this plant, there were no significant differences in the activities of SOD and/or GPX of stems as compared with roots or leaves. However, there were higher activities in CAT of leaves, stems and then in roots in both zones.

The evaluated levels of oxidative damage biomarkers of lipids such as MDA and dityrosine are shown in Table 5. Both parameters were insignificantly higher in tissues of these species that were grown in zone 1 as compared to the same plants in zone 2. In all studied plants, the levels of these biomarkers were higher in roots, stems and leaves, in both zones. There were significant differences between roots and leaves for these parameter levels.

Table 6 presents the results of relation analysis between Mn of tissues and antioxidative enzyme activities in plant species growing on zone 1. All correlation values were positive in our plant species among which *D. stramonium* and *C. ambrosioides* showed higher values with respect to *A. camelthorn*. As comparison, most of correlation values for leaves of *D. stramonium* and/or *C. ambrosioides* were greater than their roots. The correlation coefficients (r) for relationships between total plant Mn and soil pH levels were negative values for each studied plant among which *D. stramonium* was more negative than other species. This coefficient was positive when the relationship was evaluated between soil available Mn and total plant Mn.

4. Discussion

Mn toxicity is one of the most serious natural environmental problems in Mn mines, limiting plant growth and development by disturbing the metabolism in many ways. Because of different degrees of tolerance to high level of Mn, only special plants can be adapted to the condition of Mn mine.

The processes of Mn phytoavailability are affected by a variety of soil chemical and physical parameters. In this investigation, there were no significant differences in the studied soil element composition and chemical characteristics between the two zones (in and out of Mn mine),

Table 4
Antioxidant enzyme activities in different plant tissues[a]

Plant	Enzyme	Zone 1			Zone 2		
		Leaf	Stem	Root	Leaf	Stem	Root
D. stramonium	SOD (U/mg protein)	35.19[b,c]±3.23	28.64[c]±3.02	25.21[c]±2.84	14.23[b]±2.57	9.83±2.31	7.51±1.73
	CAT (μmol/min/mg)	81.47[b,c]±6.32	60.35[c]±5.73	52.61[c]±5.14	28.44±2.61	35.36±3.43	18.42±2.21
	GPX (U/mg protein)	10.82[b,c]±2.27	7.73[c]±1.74	5.22[c]±1.26	5.71±1.34	4.14±1.15	2.80±0.86
A. camelthorn	SOD (U/mg protein)	8.67[b]±1.91	6.33±2.24	5.71±1.93	6.11±1.85	3.65±1.14	4.17±1.42
	CAT (μmol/min/mg)	29.36[b]±3.73	23.10±3.20	20.22±3.43	23.14[b]±3.16	17.68±2.83	14.52±2.51
	GPX (U/mg protein)	4.83±0.92	3.14±0.87	3.91±0.84	3.64±0.82	1.94±0.38	2.37±0.75
C. ambrosioides	SOD (U/mg protein)	48.35[b,c]±4.61	31.62[c]±3.67	25.63[c]±3.25	19.34±3.13	10.82±1.94	14.51±2.56
	CAT (μmol/min/mg)	94.74[b,c]±6.51	65.63[b,c]±5.83	50.34[c]±5.35	34.25[b]±3.62	27.53±2.83	22.14±2.14
	GPX (U/mg protein)	8.15[c]±1.87	6.32[c]±1.14	5.12[c]±1.22	2.17±0.52	1.43±0.38	1.15±0.31

[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.

Table 5
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
D. stramonium	13.42±2.26	14.16±2.61	21.31[b]±3.23	0.86±0.19	1.08±0.23	1.36[b]±0.31	9.78±1.68	12.25±2.19	15.46[b]±2.78	0.72±0.17	0.89±0.21	1.07±0.26
A. camelthorn	10.18±2.73	16.30±3.07	19.22[b]±3.28	1.07±0.23	1.67±0.28	1.92[b]±0.37	7.51±1.74	12.12±2.48	14.73[b]±2.86	0.93±0.19	1.27±0.23	1.52[b]±0.29
C. ambrosioides	24.37±2.62	27.18±3.34	33.46[b]±3.83	2.11±0.27	2.81±0.24	3.24[b]±0.43	18.42±2.16	21.17±2.41	26.31[b]±3.12	1.86±0.18	2.18±0.26	2.64[b]±0.31

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

Table 6
Correlation coefficients (r) for relationships between Mn from plant tissues and other parameters of plant species and soil samples in zone 1

Parameter	R		
	A. camelthorn	*D. stramonium*	*C. ambrosioides*
Roots			
SOD–Mn	0.21	0.38	0.43
CAT–Mn	0.13	0.36	0.31
GPX–Mn	0.18	0.23	0.26
Leaves			
SOD–Mn	0.24	0.41	0.53
CAT–Mn	0.18	0.35	0.39
GPX–Mn	0.11	0.28	0.3126
Soil pH−total plant Mn	−0.36	−0.68	−0.51
Soil available Mn−total plant Mn	0.46	0.38	0.64

except Mn concentration. The ratio of total Mn in zone 1 to that in zone 2 was around 10 and was about 12 for available Mn. Accordingly and based on our results, the rate of available concentration of Mn for plants in the soils of zone 1 was considerable (around 61%), even though both sample sets had similar pH and their EC, clay contents, CEC and water holding capacity did not vary significantly.

Based on the EC (in dsm^{-1}) levels, Boulding (1994) classified the soils as: non-saline <2; moderately saline 2–8; very saline 8–16; extremely saline >16. There were no problems with soil salinity in our study zones. The low pH of the soil can increase Mn solubility and thus accelerate its release from the soil phase. In addition, preliminary study showed that Mn uptake by roots is in relation to pH values of the soil (Batty et al., 2000). To examine the influence of these parameters on Mn phytoavailability, correlations between selected soil properties and the plants Mn levels were obtained. Our statistical analysis revealed that total plant Mn concentration in each species only correlated with soil pH, and with available Mn contents of soil samples.

Accordingly, because of the similar soil pH, the availability and uptake of Mn in and out of the Mn mine could not affect Mn toxicity for the studied species. Depending on plant species, the distribution level and accumulation of excess Mn vary in different tissues. In plants growing on normal soils, Mn content in their dry

matter fall within wide range of 20–500 μg/g. Due to this range, plants grown on zone 1 contained considerably higher Mn in their tissues than normal levels. Furthermore, an operational definition was proposed by Baker (1981): in accumulator plants, the metal contents in shoots are invariably greater than in roots, showing a special ability of the plants to absorb and transport metals and store them in their above-ground parts (Reeves and Baker, 2000). In accordance to these documents, our study plants in Mn mine showed several fold increase in concentration of Mn with respect to the plants outside the mine zone. Of this increase most Mn was accumulated in their above-ground parts, particularly their leaves. Another indication was also reported by Lidon (2001), in which Mn accumulated mostly in the leaves of rice under excess Mn in nutrient solution. Evidently, our observed differences in Mn bioconcentration between plant species may primarily be due to variation in the amount of uptake. As comparison, our *C. ambrosioides*, collected from Mn mine revealed leaf Mn level (3251 ± 134 μg/g dw) slightly higher than the same collected plant species (2489 ± 107 μg/g dw) growing on Xiangtan Mn mine in China (Xue et al., 2004). In agreement with our results, all studied plant species in Xiangtan mine showed high accumulation levels of Mn in their leaves and then in stems and roots, although maximum level of soil Mn was around 114,000 μg/g, slightly lower than Mn in our copper mine (127,215 μg/g).

In excess Mn exposure, Mn toxicity could generate oxidative stress through direct generation of ROS from Mn ions in the Fenton reaction (Lynch and Clair, 2004) and direct transfer of electrons in single electron reaction, leading to a rise in ROS level. In this way the degree of cell damage or plant symptoms progression depends on the rate of ROS formation and on the efficiency and capacity of detoxification and repair mechanisms (Demirevska-Kepova et al., 2004).

Toxicity symptoms attributed to Mn uptake that has been reported by different authors were included as brown spots on mature leaves, intervenial chlorosis and necrosis, deformation of young leaves, leaf tip burning in carnation (Lidon et al., 2004). In the current study, no visual symptoms of Mn toxicity were observed in plants, suggesting resistance and/or tolerance to Mn toxicity.

For cells and tissues, to avoid or limit damages in an oxidative stress condition, elaborate antioxidant enzyme systems are necessary. Among the most important enzymes, accumulation of superoxide radical is limited by SOD, whereas, hydrogen peroxides can be scavenged by CAT or GPX (James and Murali, 2000). Accordingly, we detected not only a base line level of these enzymes in plants of zone 2, but also a marked increase in each enzyme activity was observed in plant tissues of zone 1. However, enhanced enzyme activities in the current study is consistent with other reports as they have shown that these enzymes are triggered by ROS following exposure to oxidative stress (James and Murali, 2000; Shikanai et al., 1998). In addition, our testing for relationship showed positive correlation between Mn levels in plant tissues and antioxidative enzyme activities.

This evidence supports that the elevation in enzyme activities may be attributed to Mn-induced oxidative stress in *D. stramonium* and *C. ambrosioides*. On the other hand, we had low increase in GPX with respect to high increase of CAT activities in some tissues of plants in zone 1, which could be the consequence of comparison effects of these enzymes on ROS catalysis. This concept has been reported as an activity balance mechanism for similar oxidative stress conditions (Demirevska-Kepova et al., 2004). The mechanism might be critical for maintenance of appropriate antioxidative enzyme activities in response to the elevated level of ROS (Feierabend et al., 1992).

When plants are subjected to oxidative stress, lipid peroxidation and proteins damages occur and this results in a significant accumulation of tissue MDA and dityrosine (Halliwell and Gutteridge, 1998b; Zhou et al., 1997). Our evaluations of these oxidative damage biomarkers did not show significant increase in the tissues of studied plants even in zone 1. On the basis of these observations, antioxidative enzymes probably play a key role in defense system against oxidative damages in *D. stramonium* and *C. ambrosioides*. In addition, high levels of MDA and dityrosine in roots with respect to leaves of all studied plants may be attributed to considerably low activities of antioxidative enzyme in roots as compared with leaves.

Since, no significant increases in total SOD, CAT and GPX activities were detected in *A. camelthorn* in zone 1, we suggest that another strategy was involved in this plant to increase the internal tolerance to Mn toxicity. Many studies on intrinsic tolerance mechanisms are available and suggest immobilization of excess Mn or its storage and accumulation in vacuoles (McCain and Markley, 1989) Golgi vesicles and cell walls (Hughes and Williams, 1988) as the main detoxification mechanisms. In accordance with these documents our results showed around 45% accumulation of the total Mn in the leaves vacuoles of *A. camelthorn* plant in zone 1, which is markedly higher with respect to the other two plant species. It is our conviction that this storage mechanism is considerably involved in *A. camelthorn* tolerance against excess Mn.

To sum, the present data have shown that antioxidative enzymatic response to Mn stress gets activated in *D. stramonium* and *C. ambrosioides* and possibly accumulation of Mn in leaf vacuoles of *A. camelthorn* protected them from oxidative damages and increased their tolerance to Mn excess.

Acknowledgment

Grant of the Research Deputy of Tarbiat Moalem University supported this research.

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