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Antioxidative Responses and Metal Accumulation in Invasive Plant Species Growing on Mine Tailings in Zanjan, Iran[*1]

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ABSTRACT

Tailings of a Pb and Zn mine as a metal-contaminated area (Zone 1) with two pioneer plant species, *Peganum harmala* and *Zygophyllum fabago*, were investigated and compared with a non-contaminated area (Zone 2) in the vicinity. Total concentrations of Pb, Zn, and Cu in the soil of Zone 1 were 1 416, 2 217, and 426 mg kg^{-1}, respectively, and all exceeded their ranges in the normal soils. The soil pH was in the neutral range and most of the physical and chemical characteristics of the soils from both zones were almost similar. The species *Z. fabago* accumulated higher Cu and Zn in its aerial part and roots than the normal plants. On the other hand, their concentrations did not reach the criteria that the species could be considered as a metal hyperaccumulator. The species *P. harmala* did not absorb metals in its roots; accordingly, the accumulation factor values of these metals were lower than 1. The contents of chlorophyll, biomass, malondialdehyde, and dityrosine in these two species did not vary significantly between the two zones studied. In Zone 1, leaf vacuoles of *Z. fabago* stored 35.6% and 43.2% of the total leaf Cu and Zn, respectively. However, in this species, the levels of phytochelatins (PCs) and glutathione (GSH) and antioxidant enzyme activities were significantly higher in Zone 1 than in Zone 2. In conclusion, metal exclusion in *P. harmala* and metal accumulation in *Z. fabago* were the basic strategies in the two studied pioneer species growing on the metal-contaminated zone. In response to metal stress, elevation in antioxidant enzyme activities, increases in the PCs and GSH levels in the aerial parts, and metal storage within vacuoles counteracted each other in the invasion mechanism of *Z. fabago*.

Key Words: accumulation factor, antioxidant enzymes, glutathione, metal stress, phytochelatins

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INTRODUCTION

Mining activities often generate high amounts of contaminated soils and wastes; usually deposits outside the mine area can cause deleterious effects on the soils (Dudka and Adriano, 1997). Negative impact of the mining activities on the surroundings is mainly due to the presence of high volumes of tailings. Release of metals from mine sites takes place mainly through drainage and erosion of waste dumps. These tailings usually have unfavorable conditions to most plant species growing on them, such as low pH (Obrador *et al.*, 2007), toxic metal concentrations (Norland and Veith, 1995), low water retention capacity (Henriques and Fernandes, 1991), and low levels of plant nutrients (Wong, 2003). Accordingly, the natural colonization of mine areas and tailings is usually slow.

Nevertheless, some tolerant plant species can spread easily in these environments due to the lack of competitors and potent tolerance mechanisms (Macnair, 1987). Therefore, the plant communities that can be found on mine tailings are frequently formed by few plant species (Conesa *et al.*, 2006). On the other hand, in arid and semi-arid mining zones, the establishment of vegetation also requires plant species adapted to drought. As a whole, these plants develop specific physiological and biochemical mechanisms that enable them to function normally on lands polluted with heavy metals, forming heavy metal-resistant populations (Shu *et al.*, 2002). They respond by exclusi-

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on, indication, or accumulation of toxic metals (Baker and Proctor, 1990). They may also develop scavenging systems controlling reactive oxygen species (ROS) by both non-enzymatic anti-oxidants (*e.g.*, glutathione, ascorbate, and carotenoids) and an enzymatic anti-oxidative system. The main anti-oxidative enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Kantol *et al.*, 1988).

Accordingly, the metal-tolerant plants are not only of scientific interest, but they can be used as a phytoremediation technology for the removal of metals in soil remediation. Some of these populations may belong to invasive plant species colonized on mine soils. However, there is still little information about their strategies against metal pollution and toxicity.

Two invasive plant species, *Zygophyllum fabago* and *Peganom harmala*, have naturally colonized all land around the Anguran Mine, Zanjan City, Iran, where Pb and Zn were extracted. *Zygophyllum fabago* from *Zygophyllaceae* family, a perennial erect glabrous herb, is considered as an invasive weed species (Melendo *et al.*, 2002). *Peganum harmala*, also known as Harmal or Syrian rue, is a perennial herbaceous, glabrous plant, originated in central Asia, and grows in semi-arid conditions. It has been used as a traditional herbal remedy (Sincich, 2002; Pieroni *et al.*, 2005). The aim of this study was to find the status of metals within tissues of the species and characterize the biochemical mechanisms which protected them against heavy metal toxicity.

MATERIALS AND METHODS

This study was carried out at the Anguran Mine located in Zanjan City (34° 37′ N, 47° 24′ E), Iran. It is one of the historical mining centers with extraction of Pb and Zn. The climate of the mine district is generally mild and characterized as semi-arid, with maximum temperature occurring in June and July in the range of 38–43 °C. The average annual precipitation is 347 mm. Large areas were tailings, caused by continuous ore extraction. Contaminated soils stretch along several kilometers, affecting the environment in the close vicinity. Tailings as the metal-contaminated field (Zone 1) and another field in the vicinity without any mining activity as the non-contaminated zone (Zone 2) were selected for the study.

The vegetation in the study area was dominated by two main natural species, *Zygophyllum fabago* and *Peganom harmala*. Plant samples were collected at a determined time of a single growing season. They were at the same age and collected before the flowering period. Expert botanists personally 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 in triplicate were used for analysis. The tissues, particularly roots, were cleaned in abundant deionized fresh water and rinsed with distilled water. Corresponding soil samples were collected at the location of plant sampling to a maximum depth of about 30 cm. These samples were then air-dried and sieved through a 2-mm plastic sieve.

A hydrometric method was used to analyze soil particle-size distribution (Day, 1965). Water-holding capacity (WHC) at an atmospheric pressure of 3.3 mPa was determined as the amount of water held in soil in the upper ring 24 h after drainage of water (Wang, 1989). The dried soil samples were digested with HCl + HNO_3 + $HClO_4$ (3:1:1, v/v/v) (Yuan, 1988). Metals in this acid-digested extract were determined by atomic absorption spectrometry. The metals in the soil samples were also sequentially extracted following a slightly modified method of Tessier *et al.* (1979). The modification consisted of initial extraction with double-distilled water: 2 g soil samples were shaken for 4 h in distilled water of electric conductivity < 0.02 dS m^{-1}, followed by centrifugation for 10 min at 3 000 r min^{-1}. This step extracted the fraction that is water-soluble and most easily available to plants and easily leached into the groundwater (Siebe, 1995). Then, the extract (0.1 g) was separately ashed at 550 °C and the residue was dissolved in 200 mL L^{-1} HCl. Metal contents were determined directly by atomic absorption spectrometry. The pH and electrolytic conductivity (EC) were determined in a water-soil (1:1) extract using a Beckman pH-meter and a conductivity meter (Model HI8633, Hanna Instruments Co., Italy), respectively. The electric conductivity (dS m^{-1}) of the soil samples were classified by the Boulding criteria: $<$ 2, non-saline; 2–8, moderately saline; 8–16, very saline; $>$ 16, extremely saline (Boulding, 1994).

Plant protein content was determined by the method of Bradford (1976), with standard curves prepared using bovine serum albumin (BSA). For chloroplast isolation, fresh and mature leaves (5 g) were homogenized for 15 s with a homogenizer in 50 mL pH 7.5 ice-cold grinding medium containing 0.33 mol L^{-1} sorbitol, 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 10 g kg^{-1} BSA, 2 mmol L^{-1} sodium ascorbate, and 50 mmol L^{-1} K_2HPO_4. The homogenate was

filtrated and centrifuged for 1 min at 1 000 *g* at 4 °C to remove whole cells and cell debris. The intact chloroplasts were pelleted through centrifugation at 4 500 *g* for 30 s and were gently resuspended in the same buffer without BSA and centrifuged again under the same conditions. This washing procedure was repeated twice and pelleted chloroplasts were isolated (Rusina *et al.*, 2004).

Plant vacuole isolation was achieved by the method of Kringstad *et al.* (1980). Leaves were washed in distilled H_2O, sliced, and incubated in a medium containing 0.7 mmol L^{-1} mannitol, 50 mmol L^{-1} NaOH (pH 5.5), 5 mmol L^{-1} $MgCl_2$, 2.00 g L^{-1} cellulysin (Calbiochem, USA), 10 g L^{-1} pectinase (Sigma, USA), and 1 g L^{-1} BSA. Tissue digestion was carried out at 30 °C for 30–45 min. The digested leaf tissue was filtered and washed with 25 mL of a buffer containing 0.5 mol L^{-1} mannitol, 25 mmol L^{-1} Tris-HCl, and 5 mmol L^{-1} ethyleneglycol tetraacetic acid (EGTA). The protoplasts were collected and resuspended in 40 mL of resuspension buffer. Vacuoles were isolated from protoplast on 10 mL discontinuous Ficoll-400 gradients of 50, 100, and 150 g L^{-1}. The protoplast suspension (1 mL) was gently layered onto the top of the Ficoll-400 gradient. Each Ficoll solution was made up in 0.5 mol L^{-1} mannitol and 25 mmol L^{-1} Tris-HCl (pH 8.0). The gradients were centrifuged at 26 000 r min^{-1}. The vacuoles were removed from the gradient with a 16-gauge cannula attached to a 1-mL syringe and setting in a standard hypotonic solution.

Chlorophyll and biomass were determined by extracting the fresh and mature leaves (0.5 g) with 10 mL 800 g L^{-1} acetone as described by Alan (1994). The absorbance of the extract was measured at 663 and 645 nm in an ultraviolet (UV)-visible (Vis) light spectrophotometer (Model UV-9100). The chlorophyll content (C_T) was calculated using the equation as follows:

$$C_T = 20.2A_{645} + 8.02A_{663} \quad (1)$$

where A_{645} and A_{663} are the absorbance at 663 and 645 nm, respectively. The clean plants were separated into roots and shoots and dried on an oven at 60 °C for 48 h, and then the biomass (on dry weight basis) was measured and recorded as mg g^{-1} fresh weight (mg g^{-1} FW).

Extraction and analyses of phytochelatins (PCs) and glutathione (GSH) were performed according to the method described by Sneller *et al.* (2000) with a slight modification. Frozen plant tissues were homogenized in a mortar and pestle with quartz sand in 2 mL of 6.3 mmol L^{-1} diethylenetriaminepentaacetic acid (DTPA) with 1 mL L^{-1} trifluoroacetic acid (TFA) at 4 °C. The homogenate was centrifuged at 14 000 *g* at 4 °C for 12 min. The clear supernatants were collected for the assay by high-performance liquid chromatography (HPLC) after pre-column derivatization with a fluorescent probe, monobromobiane (mBrB). An amount of 250 μL of the supernatant was mixed with 450 μL of 200 mmol L^{-1} 3-[4-(2 hydroxyethyl)-1-piperazinyl] propanesulfonic acid buffer substance (HEPPS) at pH 8.2, 6.3 mmol L^{-1} DTPA, and 10 μL of 25 mmol L^{-1} mBrB. Derivatization was carried out in the dark at 45 °C for 30 min. The reaction was terminated by adding 300 μL of 1 mol L^{-1} methanesulfonic acid (MSA). The samples were stored in the dark at 4 °C until HPLC analysis. Blank samples were used to identify the reagent peaks. The bimane derivatives were separated using a binary gradient of mobile phase A (1 mL L^{-1} TFA) and B (pure acetonitrile) at room temperature (22 ± 2 °C). Fluorescence was detected at 380 nm excitation and 470 nm emission wavelengths. The flow rate was 0.5 mL min^{-1}. The derivatives (50 μL) were run in a linear gradient from 120 to 250 g L^{-1} mobile phase for 15 min, then from 250 to 350 g L^{-1} mobile phase for 14 min, and finally from 350 to 500 g L^{-1} mobile phase for 21 min. Before injecting a new sample, the column was cleaned (for 5 min, 1 000 g L^{-1} mobile phase) and equilibrated (10 min, 10 g L^{-1} mobile phase) with a post-equilibration time of 5 min. Total analysis time was 70 min. Analytical data were integrated using the HP ChemStation. Retention times of the PCs and GSH in the biological samples were checked with those of the standard solutions of PCs and GSH, respectively. Individual PC subtypes were quantified using the relationship peaks *vs.* concentrations of GSH standard solutions. Corrections for differential derivatization efficiencies were made according to the method stated by Sneller *et al.* (2000).

For measurement of dityrosine in the plant samples, 1.2 g fresh tissue materials were homogenized in 5 mL 50 mmol L^{-1} ice-cold HEPES-KOH buffer (pH 7.2) containing 10 mmol L^{-1} EDTA, 2 mmol L^{-1} phenylmethanesulfonyl fluoride (PMSF), 0.1 mmol L^{-1} *p*-chloromercuribenzoic acid, 0.1 mmol L^{-1} DL-norleucine, and 100 mg Polyclar AT. The homogenate was centrifuged at 5 000 *g* for 60 min to remove debris. Purification of *o*,*o*′-dityrosine in the homogenized supernatant fluid of the clear tissues was accomplished by 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 the eluent varied linearly from 1:99:0.02 to 20:80:0.02 acetonitrile-water-TFA over 25 min. A flow rate of 4 mL min^{-1} was used. *o,o′*-dityrosine was analyzed by a reversed-phase HPLC column with simultaneous UV detection (280 nm) and fluorescence detection (excitation wavelength: 280 nm; emission wavelength: 410 nm). A Phenomenex Inertsil ODS-2 HPLC column (150 mm × 4.6 mm, 5 μm, Bester, the Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mmol L^{-1} ammonium acetate (pH 4.5). The flow rate was 0.8 mL min^{-1}. A standard dityrosine sample was prepared according to Amado *et al.* (1984). Dityrosine was quantified with horseradish peroxidase in the presence of H_2O_2 (extinction coefficient of 4.5 mmol L^{-1} cm^{-1} at pH 7.5).

For malondialdehyde (MDA) analysis of the plant samples, proteins of the tissue homogenate were precipitated with 400 g L^{-1} trichloroacetic acid (TCA). The MDA assay was based on the reaction of malondialdehyde with thiobarbituric acid (TBA) 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 an HP 1050 gradient pump (Hewlett-Packard Co., USA) equipped with an automatic injector, a 1050 diode-array absorption detector, and a personal computer using the ChemStation Software from the Hewlett-Packard Co., USA. Aliquots of the TBA + MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 mm × 4.6 mm). The mobile phase consisted of 150 g L^{-1} methanol in degassed double-distilled water. The flow rate was 2 mL min^{-1}. The MDA + TBA standards were prepared using tetraethoxypropane. The absorption spectra of the standards and samples were assayed at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the sample protein content.

For determining enzyme activities of the plant samples, whole tissues (leaves, stems, and/or roots) were homogenized (1:5, w/v) separately in a 50 mmol L^{-1} pH 7.0 sodium phosphate buffer containing 1 mol L^{-1} NaCl, 10 g L^{-1} polyvinylpyrrolidone, and 1 mmol L^{-1} EDTA in an ice-cold mortar. After centifugation at 20 000 *g* for 15 min, the supernatant (crude extract of leaves) was used to determine enzyme activities at 25 °C.

Catalase (EC 1.11.1.6) activity was determined after consumption of H_2O_2 (extinction coefficient of 0.0394 mmol L^{-1} cm^{-1}) at 240 nm for 30 s (Aebi, 1984). The assay mixture contained 100 mmol L^{-1} potassium phosphate buffer (pH 7.0), 15 mmol L^{-1} H_2O_2, and 50 μL leaf extract in a 3-mL volume. The unit of the catalase activity was defined as nmol L^{-1} H_2O_2 decomposed per 1 min. The method of Hopkins and Tudhope (1973), with butyl hydroperoxide as a substrate, was used to detect glutathione peroxidase (GSH-Px) (EC 1.11.1.9) activity. The reaction mixture comprised 50 mmol L^{-1} potassium phosphate buffer (pH 7.0), 2 mmol L^{-1} EDTA, 0.28 mmol L^{-1} nicotinamide adenine dinucleotide phosphate (NADPH), 0.13 mmol L^{-1} GSH, 0.16 unit glutathione reductase (GR), 0.073 mmol L^{-1} *t*-butyl hydroperoxide, and the extract (50 mg protein). One unit of GSH-Px activity was defined as the amount of enzyme that catalyzed the oxidation of NADPH (mmol L^{-1} min^{-1} mg^{-1} protein). Superoxide dismutase activity was determined by the method of Minami and Yoshikawa (1979) with 50 mmol L^{-1} Tris-cacodylic sodium salt buffer (pH 8.2) containing 0.1 mmol L^{-1} EDTA. The reaction mixture was composed of 14.2 g kg^{-1} Triton X-100, 0.055 mmol L^{-1} nitroblue tetrazolium (NBT), 16 mmol L^{-1} pyrogallol, and the extract (50 mg protein). The unit of the superoxide dismutase activity (50% inhibition) was established according to the definition of McCord and Fridovich (1969) and defined as the quantity of the enzyme required to inhibit the reduction of NBT by 50% per 1 min.

The translocation factor (TF), enrichment coefficient (ECo), and accumulation factor (AF) of heavy metals (Zu *et al.*, 2005) were calculated as follows:

$$\text{AF} = \text{element in shoot/normal level in plant} \quad (2)$$

$$\text{TF} = \text{element in shoot/element in root} \quad (3)$$

$$\text{ECo} = \text{element in shoot/available level in soil} \quad (4)$$

All statistical analyses were carried out using procedures available in the SPSS v.10 (SPSS Inc., USA) statistical package. We calculated means ± standard deviations of 3 replicates of the test results. Student's *t*-test was applied to determine the significance of results between different samples. Statistical significance was set at the $P < 0.05$ level. Pearson's correlation coefficients were calculated to test the relationships between parameters.

RESULTS

Most physical and chemical characteristics of the

soils collected from the two zones in this study were almost similar and/or their differences were not remarkable (Table I). The levels of Co, Mn, and Ni were generally below the maximum allowable concentrations of heavy metals in soil (Bowen, 1979). However, in Zone 1, total and/or available levels of Pb, Cu, and Zn exceeded the ranges in normal soils and this zone was considered as a contaminated zone. Since the soil EC values of both zones varied in the range of 0–2 dS m^{-1}, the soils were non-saline and there were no considerable problems of soil salinity (Boulding, 1994). On the other hand, the range in which pH varied was in the neutral category as determined by the criteria of soil pH classes (Tanji, 1990).

Table II revealed the distribution levels of toxic heavy metals in roots, leaves, and stems of the plant species studied and their translocation and accumulation factors within tissues. For *Zygophyllum fabago* collected from Zone 1, the levels of Cu and Zn in the aerial part (shoots) and roots exceeded their normal concentration ranges (Reeves and Baker, 2000); the TF values for Pb, Cu and Zn were 2.14, 5.63, and 9.30, respectively, but ECo and AF values were greater than 1 for Zn and Cu. On the contrary, Pb was at a normal concentration in this plant species collected from each of the studied zones. These heavy metals were also below the criteria for plants to be considered as a hyperaccumulator. Among different plant parts, the leaves as the main part accumulated most of the evaluated heavy metals. In *Peganum harmala*, the toxic metals were at levels higher than the normal values in plants. The ECo and AF values for *Peganum harmala* were lower than 1 in each studied zone. However, the TF values were greater than 1 in both zones.

The biomass of the plant species and chlorophyll contents in their leaves are given in Table III. For *Zygophyllum fabago* from Zone 1, the biomass of shoots was insignificantly larger than that of Zone 2; however there was insignificant decrease in the biomass of its roots. The chlorophyll contents of leaves of this plant species and *Peganum harmala* in Zone 1 were also insignificantly higher than those of Zone 2 ($P < 0.05$). On the other hand, the biomass levels of shoots and roots of *Peganum harmala* collected from Zone 1 were insignificantly decreased as compared with those of Zone 2.

The levels of metals in the isolated vacuoles and chloroplasts from the plant leaf tissues are presented in Table IV. In general, both species in Zones 1 and 2 had lower than 10% metals in their chloroplasts with unremarkable differences between zones. *Peganum harmala* collected from Zone 1 also showed low accumulation of heavy metals in vacuoles. In Zone 1, most of Cu and Zn (35.6% and 43.2%) were accumulated in the vacuoles of *Zygophyllum fabago* leaves. In addition, the levels of PCs of 2 and 3 subunits (PC_2, and PC_3) and GSH in the tissues of *Zygophyllum fabago* were significantly higher in Zone 1 than Zone 2 (Fig. 1). There were insignificant differences between zones in the levels of these parameters for *Peganum harmala* ($P < 0.05$).

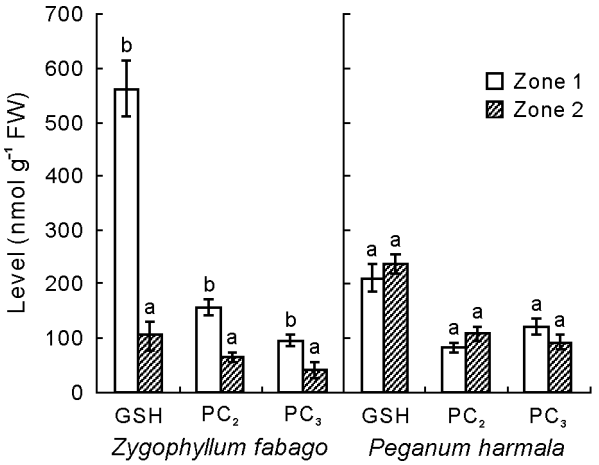

Fig. 1 Levels of reduced glutathione (GSH) and phytochelatins of 2 and 3 subunits (PC_2 and PC_3) in the tissues of the two plant species in the two zones studied. Vertical bars represent standard error of the mean. Bars with the same letter indicate no significant difference between zones at $P < 0.05$.

Antioxidant enzyme activities and oxidative damage parameters in the leaves of the two plant species are listed in Table V. The enzyme activities in the leaves of *Zygophyllum fabago* were significantly higher in Zone 1 than Zone 2 ($P < 0.05$). On the other hand, there were no significant differences in MDA and dityrosine as oxidative damage parameters between zones. However, *Peganum harmala* did not show significant differences in these parameters between Zones 1 and 2 ($P < 0.05$).

DISCUSSION

Mine tailings impose various effects on plants, such as exposure to high levels of various heavy metals and increase in absorption of other elements at toxic concentrations (Bradshow and Chadwick, 1980). In this study, tailings as a metalliferous soil were located in the vicinity of the Anguran Mine and were heavily enriched with toxic metals such as Pb, Zn, and Cu. Total levels of each of these metals were higher than their

TABLE I

Chemical composition and physical characteristics[a)] of soils of the two zones studied

Zone	Pb		Zn		Mn		Cu		Co	
	Total	Available	Total	Available	Total	Available	Total	Available	Total	Available
	mg kg^{-1} DW									
1	1 416.3±139[b)] b[c)]	548.2±47.1 b	2 217±194 b	687.2±63.2 b	33.2±4.8 a	5.64± 0.51 a	426.3±32.5 b	123.5±10.6 b	29.2± 3.5 a	4.61±0.42 a
2	84.3±7.2 a	30.2±3.31 a	317±27 a	95.2±10.2 a	24.8±3.9 a	5.21±0.42 a	207.5±16.7 a	51.3±4.6 a	23.9±2.6 a	3.93±0.29 a

Zone	Ni		Soil texture			EC	pH(H_2O)	WHC
	Total	Available	Sand	Slit	Clay			
	mg kg^{-1} DW		g kg^{-1}			dS m^{-1}		m^3 m^{-3}
1	97.6±8.1 a	23.8±1.94 a	330	290	380	1.47±0.18 a	6.6±0.33 a	0.28±0.04 a
2	112.5±7.4 a	20.1±1.52 a	260	320	420	1.13±0.15 a	7.2±0.28 a	0.36±0.05 a

[a)]EC = electrolytic conductivity in water-soil extract (1:1); WHC = water-holding capacity.
[b)]Mean±standard deviation.
[c)]Means followed by the same letter within a column are not significantly different ($P < 0.05$).

TABLE II

Levels of heavy metals and their translocation factor (TF), accumulation factor (AF), and enrichment coefficient (ECo) values in tissues of two plant species in the two zones studied

Plant species	Heavy metal	Zone 1							Zone 2						
		Leaf	Stem	Root	Shoot	AF	TF	ECo	Leaf	Stem	Root	Shoot	AF	TF	ECo
		mg kg^{-1}							mg kg^{-1}						
Zygophyllum fabago	Pb	12.43±0.93[a)]	4.31±0.32	7.82±0.61	16.74±1.26	0.67	2.14	0.1	5.93±0.38	3.10±0.21	5.24±0.48	9.03±0.66	0.36	1.72	0.29
	Cu	317.2±24.3	121.5±9.6	77.8±7.1	438.7±35.2	17.5	5.63	3.54	7.86±0.61	2.83±0.19	6.10±0.55	10.69±1.24	0.42	1.75	0.20
	Zn	418.6±33.5	142.3±12.6	60.4±6.2	560.8±51.2	14.02	9.3	1.5	11.14±0.88	4.50±0.36	3.71±0.31	15.64±1.12	0.39	4.21	0.16
Peganom harmala	Pb	10.52±1.31	8.43±0.72	3.40±0.21	18.95±1.81	0.76	5.57	0.11	7.14±0.59	6.20±0.66	3.82±0.24	13.37±1.10	0.53	3.50	0.44
	Cu	5.38±0.42	2.41±0.18	3.96±0.33	7.79±0.54	0.31	1.96	0.06	4.10±0.33	2.17±0.20	2.66±0.18	6.27±0.54	0.25	2.35	0.12
	Zn	19.51±1.85	7.84±0.66	10.38±1.15	27.30±2.84	0.68	2.65	0.07	9.34±0.81	5.12±0.46	7.40±0.68	14.46±1.52	0.36	1.95	0.15

[a)]Mean±standard deviation.

TABLE III

Chlorophyll contents and biomass of plant tissues in the two zones studied

Plant species	Zone 1			Zone 2		
	Biomass		Chlorophyll	Biomass		Chlorophyll
	Shoot (stem + leaf)	Root		Shoot (stem + leaf)	Root	
	mg g^{-1} FW					
Zygophyllum fabago	58.12±4.58[a)]	35.68±3.87	2.14±0.28	49.88±4.24	44.14±4.57	2.68±0.23
Peganom harmala	85.79±5.85	69.85±4.79	1.37±0.18	97.21±6.69	81.11±6.33	1.78±0.25

[a)] Mean±standard deviation.

TABLE IV

Heavy metals stored in organelles as a percentage of the total heavy metal content in the plant leaves collected from the two zones studied

Plant species	Heavy metal	Zone 1		Zone 2	
		In vacuoles	In chloroplasts	In vacuoles	In chloroplasts
		%			
Zygophyllum	Pb	3.8	5.2	5.1	4.4
fabago	Cu	35.6	8.7	6.3	8.2
	Zn	43.2	9.3	3.8	5.7
Peganom	Pb	4.2	7.7	5.3	6.8
harmala	Cu	8.8	5.4	9.2	4.1
	Zn		9.2	8.7	8.0

TABLE V

Activities of antioxidant enzymes and oxidative damage biomarkers in leaves of the plant species collected from the two zones studied

Plant species	Zone 1					Zone 2				
	Superoxide dismutase	Catalase	Glutathione peroxidase	Mlondialdehyde	Dityrosine	Superoxide dismutase	Catalase	Glutathione peroxidase	Mlondialdehyde	Dityrosine
	units mg^{-1} protein			nmol mg^{-1} protein		units mg^{-1} protein			nmol mg^{-1} protein	
Zygophyllum	262.8±	124.2±	44.8±	28.43±	66.36±	81.7±	52.5±	15.5±	23.66±	54.69±
fabago	6.4[a)] b[b)]	8.8 b	3.7 b	3.05 a	6.78 a	2.1 a	3.8 a	1.1 a	2.70 a	4.96 a
Peganom	78.1±	188.2±	96.83±	51.42±	28.86±	87.2±	163.9±	113.6±	42.50±	36.06±
harmala	4.3 a	14.5 a	8.7 a	4.72 a	3.49 a	5.3 a	12.6 a	10.1 a	4.81 a	3.95 a

[a)] Mean±standard deviation.
[b)] Means followed by the same letter are not significantly different ($P < 0.05$) for an enzyme or a biomarker between zones.

concentration than their concentration ranges in normal soils. The Pb concentration exceeded 1.4 times the maximum admissible in soils by the Italian legislation for industrial use (1 000 mg kg^{-1}), 2.8 times the Dutch intervention level (530 mg kg^{-1}). The concentrations of Zn were about 2 times higher than those of Pb. In addition, the Zn levels were 3 times higher than the Dutch intervention level (720 mg kg^{-1}) and 1.5 times higher than the maximum admissible for industrial use (1 500 mg kg^{-1}). Since the soil physicochemical characteristics of these sites were not favorable to most plant species, our two invasive plant species were endemic in mine land and grow normally without any visual and conspicuous symptoms of heavy metal toxicity. Because of their adaptation potentials, they are herb natives to arid and semiarid areas widely distributed in central Asia, Africa, Middle East, South America, Mexico, south USA, and Australia (Bellstedt *et al.*,

2008). *Zygophyllum fabago* as a pioneer plant community had colonized areas of low salinity in mine tailings and wastes in the Sierra de Cartagena (Conesa *et al.*, 2007a, b). This species in our study showed higher concentrations of Zn and Cu in Zone 1 than Zone 2, indicating that *Z. fabago* had a strong ability to tolerate high levels of these elements. The values of enrichment coefficient (ECo) and accumulation factor (AF) for Zn and Cu were greater than 1, indicating a high potential of this species to absorb and accumulate these metals. However, the species could not be considered as a metal-hyperaccumulator because the concentrations of heavy metals in the shoots did not reach the prescribed hyperaccumulation levels, Pb and Cu $> 1\,000$ mg kg^{-1} (Baker *et al.*, 1994) and Zn $> 10\,000$ mg kg^{-1} (Brown *et al.*, 1994). In accordance with our findings, another research on *Z. fabago* that dominated the Cartagena-La Union Mine tailing showed accumulation of Zn, Pb, and Cu lower than threshold limits that are prescribed for hyperaccumulators (Baker *et al.*, 1994; Brown *et al.*, 1994). This species was presented as an adaptable plant cover, suitable for long-term reclamation. The values of TF for Zn and Cu were also greater than 1, which clearly illustrated that the translocation of these accumulated metals was higher from the roots to the shoots due to efficient metal transporter systems (Zhao *et al.*, 2003). Accordingly, different plant parts had different budgets of metals.

In soils, some metals such as Pb occur as insoluble precipitates (phosphates, carbonates, and hydroxyoxides), which are largely unavailable for plant uptake (Pitchell, 1999). Our study revealed an available Pb level of 410 g kg^{-1} in Zone 1; however both species showed AF and ECo values lower than 1. Accordingly, *Z. fabago* developed avoidance mechanism to prevent Pb uptake into roots, retarding translocation and accumulation in shoots. In agreement with our research, Alvarenga *et al.* (2004) demonstrated that naturally occurring vegetation in tailings in a pyrite mining area in SW Portugal accumulated Mn and excluded Pb. Therefore, *Z. fabago* might behave differently with different metals in the soil, and acted as an accumulator for Zn and Cu and as excluder for Pb. In *P. harmala*, AF and ECo values for Pb, Zn and Cu were also lower than 1, indicating that the species possessed the ability to exclude these metals instead of taking up and transporting them within the plant tissues. A similar finding was reported for *P. harmala* growing on mining sites in South Morocco, where Pb, Zn, and Cu were the main contaminants and the plant excluded these metals with the lowest TF values (Boularbah *et al.*, 2006). It is evident from the present study that *P. harmala* may be considered as a metal excluder which can restrict metal bioavailability and accumulation.

Furthermore, metal compartmentalization in the vacuoles of epidermal cells has been documented following their transportation from roots into the leaves. In this condition, metals such as Zn stored and inactivated in oxalate and/or phytate complexes in vacuoles, leading to decreases in metal toxicity (Van Steveninck, 1990). Besides, this organelle storage may be involved in metal detoxifying by decreasing the levels of metals out of vacuoles of *Z. fabago*. With regard to this observation, we found that *Z. fabago* collected from Zone 1 accumulated about 41% and 31% of Zn and Cu, respectively, markedly higher than *P. harmala*. The later species did not reveal considerable vacuole storage because of low metal contents in the aboveground part. On the other hand, both species showed low chloroplast contents of metals with insignificant variations in their storage levels in the studied zones. Consequently, low chlorophyll degradation and structural damage may be attributed to low metal exposure of this biomolecule (Devi and Prasad, 1998; Prasad *et al.*, 2001).

In support of metal detoxification, their accumulation in plants has been shown to induce the production of reduced glutathione (GSH) and then phytochelatins as thiol-rich peptides that play as a metal transporter in vacuoles (Rauser, 1995). Metal ions are firstly chelated by GSH and then transferred to PCs for eventual sequestration (Gupta *et al.*, 1998). In accordance to these findings, our results exhibited significant increases in PCs and GSH in *Z. fabago* of Zone 1 as compared to Zone 2. Synthesis of PCs and GSH is a constitutive mechanism to cope up with toxic metals in this species. On the other hand, low levels of Pb, Cu, and Zn in the tissues of *P. harmala* may lead to insignificant variations in PCs and GSH between the zones studied. Although each plant species might have a unique mechanism against metals, their biochemical responses are complex and several defense strategies including antioxidative responses have been suggested (Haque *et al.*, 2007). Several heavy metals stimulate the formation of free radicals and ROS by direct electron transfer involving metal cations, consequently increasing antioxidative enzyme activities. It is evident from many studies that the increase in *de novo* synthesis of antioxidant enzyme proteins is in response of their genes by ROS-mediated signal transductions (Fatima and Ahmad, 2004; Mishra *et al.*, 2006). On this

basis, the significant elevation in the antioxidative enzyme activities of *Z. fabago* from Zone 1 could be due to the induction of excess metals. These enzymes present in various cellular compartments, functioning at different steps of ROS degradation and removal (Mishra *et al.*, 2006). When antioxidative enzymes as a scavenging system of a plant cope well with the formation of ROS, they cause the controlled oxidation of macromolecules, which results in low levels of oxidative damage biomarkers. In accordance with these observations, low levels of MDA and dityrosine in *Z. fabago* and their insignificant variations between zones may be attributed to the high levels of antioxidative enzyme activities in this plant species collected from Zone 1. We also concluded that the elevations in activities of studied enzymes were sufficient to protect proteins, chlorophyll, and lipids of plant species against ROS attack.

In conclusion, there was seemingly a high degree of habitat specialization accompanied by multiple adaptive strategies, which provided *P. harmala* generally, and *Z. fabago* specially, the ability to occupy mine tailings. Our study suggested that metal exclusion and accumulation, both were the basic strategies, acted differently in two pioneer species, making them capable to grow in the metal-contaminated zones. In addition, the activated antioxidative enzyme activities and elevated levels of PCs and GSH, both in response to metal stress with metal storage within vacuoles, counteracted each other in the invasion mechanisms of *Z. fabago*. These two species can be used for revegetation in order to control erosion in metal-contaminated sites.

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