The role of antioxidative enzymes in copper tolerance strategy of *Mimosaceace prosopis farcta* growing in a copper mine

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Abstract

In this study, we determined the accumulation levels of copper in tissues and the status of antioxidant enzyme activities in *Mimosaceace prosopis fracta* against Cu-toxicity in a copper mine. We measured the level of chlorophyll and the activities of superoxide dismutase, glutathione peroxidase and catalase by spectrometry, malondialdehyde and dityrosine by HPLC and the levels of Cu in tissues and soils by atomic absorption spectrometry. Total and available copper were at toxic levels for plants growing in contaminated soil (zone 1). However, there were no visual or conspicuous symptoms of Cu-toxicity in plant species. Excess copper was transferred into *C. ambrosioides* tissues. The *Mimosaceace prosopis fracta* accumulated Cu in roots and then in leaves, in which the leaves' chloroplasts stored Cu to approximately two times that of vacuoles. In zone 1, the chlorophyll levels increased significantly in leaves of *Mimosaceace prosopis fracta* with respect to the same plant growing in uncontaminated soil (zone 2). The studied plants in zone 1 revealed a significant increase in tissue antioxidant enzyme activities in comparison with the same plants in zone 2. The levels of oxidative damage biomarkers of lipids, such as MDA and proteins such as dityrosine, were higher in tissues of *Mimosaceace prosopis fracta* that were grown in zone 1 as compared to the same plant species in zone 2, though this difference was not significant. 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.

We concluded that elevation of antioxidative enzyme activities was a tolerance strategy in the studied plants that protected them against copper toxicity.

Introduction

Copper (Cu), as well as other heavy metals, enter plants mainly through the root system and play essential roles in a number of physiological processes, such as Co_2 assimilation and ATP synthesis. It is a micronutrient for plants and a component of various proteins, particularly those involved in both the photosynthetic (plastocyanin) and the respiratory (cytochrome oxidase) electron transport chain.[1] Soils may contain elevated levels of copper because of enhanced industrial and mining activities in recent decades. The uptake of 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.[2]

In excess, the absorbed copper plays a cytotoxic role, induces stress and can unfavorably cause injury and symptoms to plants, including growth retardation and leaf chlorosis,[3-5] in which the role of oxidative stress and reactive oxygen species (ROS) production may be involved.[6,7] Under Cu-toxicity, excess copper is an efficient generator of ROS in Fenton-type reactions, leading to disturbance of metabolic pathways and macromolecular damage.[8] ROS such as singlet oxygen, hydrogen peroxide and hydroxyl radical are generally very reactive molecules possessing an unpaired electron, and in normal conditions the balance between the generation and diminution of ROS is controled by the antioxidant defense 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 in plant growth and development.[9,10]

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.[11] 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.[12,13] 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 eliminating or reducing the damage caused by them.[14] 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.[15] Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxified the organic peroxides; it can also act on organic hydroperoxides.[16]

Plants growing on Cu-contaminated environments may develop a variety of other defense mechanisms against its toxicity. Among plants, Cu-tolerant genotypes are better able to protect them against homeostatic disturbance and cellular damage by evoking the antioxidant enzyme induction as a general response to toxic effects of heavy metals.[17] The extent of such tolerance and degree of adaptation is a highly variable mechanism in which the efficiency and capacity of detoxification play an important role.[18] Additionally, a network of sequestration activities and immobilization functions regulate the uptake, distribution and detoxification of excess metal ions in plants.[19] In the present work, field surveys have been carried out on the plants growing in a copper mine in Kerman state. The aim of this work was to investigate the ability of a wild-type plant to accumulate Cu, growth responses, antioxidative enzyme activities and the levels of oxidative damage products of lipids and proteins to clarify some aspects of the plant's tolerance mechanism under high copper concentration.

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Materials and Methods

Copper mine area and study site

This study was carried out at Chahar Gonbad, located in Sirjan province (Longitude: 0°, 11', 56'' E, Latitude: 30°, 35', 29'' N). The rainfall was around 465 mm and there were no industries nearby. The maximum temperature was +34°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 the center of the copper mine and zone 2 was approximately 9.2 km south of a waterlogged area of the copper mine. The ecological conditions were similar in both areas. The soil of zone 2 had never been subjected to sources of Cu. The copper mine was one of the most well-known copper mines where the main activity was copper extraction. Tailing had been abandoned for 14 years at the time of sampling.

Plant and soil sampling

The plant species, *Mimosaceace prosopis fracta*, as one of the most common native wild-type and endemic plant species, grows naturally in the fields studied in the mine and in the vicinity considered for this study. Growth periods were during the same season in both zones. At each site, plant samples were collected at a specific time of a single growing season and according to the actual landform of the copper mine and the distribution of vegetation before the flowering period.

Care was taken to collect plant samples from both zones while they were at the same age of growth. We had three random regions at each zone of study. We collected at least 5-8 plants of our species from each region considered. Fresh tissues, including roots, stems and mature leaves of collected plants, were considered for three replicate analyses.

Plant species were cleaned in abundant deionised fresh water, rinsed with distilled water and personally identified by an 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 the rooting zone (maximum sampling depth approximately 30 cm) and transferred to polythene bags. Excess air was squeezed out, the bags sealed, transferred to the laboratory, and stored at 4°C for a 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.

Soil analysis

Dried soil samples were digested with HCl + HNO_3 + $HClO_4$ (3:1:1, v/v).[20] Total Cu and other metals were determined by atomic absorption spectrophotometer (AAS) (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 AAS.[21] 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, most easily available to plants and easily leachable into the groundwater.[22] Soil nitrate (NO3-) was analyzed according to the method of Primo and Carrasco.[23] The total Kjeldahl nitrogen (TKN) was determined by the method outlined in Bermen and Mulvaey.[24] A hydrometric method was used to analyze soil particle size.[25] Cation-exchange capacity was determined by a standard method.[26]

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.

Plant biomass and copper content analysis

The washed plants were separated into roots and shoots, dried in an oven at 60°C for 48 h and biomass (DW) was then 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)] and heated in 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 AAS.

Chlorophyll determination

Fresh and mature leaves (0.5 g) were extracted with 10 mL 80% acetone, as described by Alan.[27] 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 follows: $C_T = 20.2\ A_{645} + 8.02\ A_{663}$.

Chloroplast isolation

Fresh and mature leaves (5 g) were homogenized for 15 sec 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 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 sec and were gently resuspended in the same buffer without BSA and centrifuged again in the same conditions. This washing procedure was repeated twice and pelleted chloroplasts were isolated.[15]

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 π=300 mOsm with D-sorbitol. After setting the vacuoles, the hypotonic solution was carefully replaced by standard bath solution.[28]

Measurement of dityrosine

Fresh tissue material (1.2 g) was 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 5,000 g for 60 min to remove debris. Purification of *o,o´*_dityrosine in the clear tissue homogenized supernatant fluid was

Table 1. Chemical characteristics of soils of the studied zones.*

Zone	Heavy metal content [mg / (Kg dw)]										pH	E. C# (mS/cm)	Nitrogen (g/kg dw)		Clay (g/kg)	CEC (cmol/kg)
	Cu		Zn		Co		Cd		Pb				NO_3^-	Total		
	T	E	T	E	T	E	T	E	T	E						
1	1132±61.4¶	424±22.6¶	40.17±6.5¶	6.27±0.53¶	6.53±1.86	<0.19	4.26±0.87	<0.23	11.3±2.15	<0.36	5.48±0.39	2.61±0.28	0.34±0.07	1.76±0.18	19.7±4.2	283±36
2	36.18±4.42	7.30±0.86	23.12±4.6	4.36±0.41	2.83±1.15	<0.14	1.53±0.46	<0.15	7.8±1.98	<0.36	6.53±0.26	1.84±0.16	0.40±0.08	2.15±0.16	14.7±3.8	231±29

T, total content; E, DTPA-extractable content. E.C, electric conductivity. CEC, cation exchange capacity. *Data were presented as mean ± SD. ¶Significant difference with respect to zone 2 ($P < 0.05$). #Electrolytic conductivity in water: soil extract (1:1).

accomplished by preparative HPLC.

*o,o´*_dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250×10 mm).[11] 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*.[29] 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 e315 = 4.5 mM^{-1} cm^{-1} at pH 7.5).

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*.[30] 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 0.5μm filter (Millipore, Bedford, MA, USA). 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 MDA normalized to the sample protein content. Protein content was determined by the Bradford method with standard curves prepared using BSA.[31]

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 co-efficient 0.0394 mM. cm^{-1}) at 240 nm for 30 sec.[32] 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 for 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.[33] 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.[34] 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.[35] Unit was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% for 1 min.

Statistical analysis

All statistical analysis was carried out by using procedure available in the SPSS v.10

Table 2. Copper bioconcentration in tissues (mg/kg dw) and in organelle (μg/g. dw) of leaves.*

Zone	Tissue			Organelle			
	Leaf	Stem	Root	Chloroplasts	%#	Vacuoles	%#
1	88.1±10.3[b¶]	192.5±18.6[b¶]	417.6±31.1	15.85±1.35[c¶]	18	7.92±0.81	9
2	12.1±.6¶	16.2±2.3	43.6±5.1	1.82±0.24	15	1.7±0.19	14

*Data are presented as mean + SD; [b]statistically different with respect to roots; [C]significant difference as compared with vacuoles; ¶statistically different with respect to zone 2; #the rate of leaf organelle Cu to total leaf Cu (as percent).

Table 3. Chlorophyll content, and biomass of different plant tissues.*

Zone	Biomass [mg /(g FW)]		Chlorophyll
	Shoot (Stem + leaf)	Root	[mg /(g FW)]
1	73.52±5.24¶	49.11±3.75	1.06±0.12¶
2	62.15±4.32	43.22±3.51	0.71±0.08

*Data are presented as mean ± SD.

Table 4. Antioxidant enzyme activities and biomarkers of lipid peroxidation and protein oxidation in tissues of studied plants.*

	Zone	SOD (U/mg protein)	GPX (U/mg protein)	CAT (uM/min/mg)	MDA (nmol/mgprotein)	Dityrosine (nmol/mg protein)
leaf	1	48.35±4.61[#¶]	8.15±1.87¶	94.74±6.51[#¶]	10.15±2.77	1.07±0.26
	2	19.34±3.13	2.17±0.52	34.25±3.62[#]	7.50±1.74	0.98±0.19
stem	1	31.62±3.67¶	6.32±1.14¶	65.63±5.83[#¶]	16.34±3.09	1.59±0.28
	2	10.82±1.94	1.43±0.38	27.53±2.83	12.17±2.48	1.27±0.24
root	1	25.63±3.25¶	5.12±1.22¶	50.34±5.35¶	18.22±3.28¶	1.95±0.38¶
	2	14.51±2.56	1.15±0.31	22.14±2.14	14.75±2.86¶	1.54±0.29¶

*Data are presented as mean + SD; ¶significant difference as compared with zone 2; #significant difference as compared with roots.

(SPSS INC., Chicago, IL, USA) statistical package. Each experiment was run on each sample at least in three replicates. Mean ± SD of test results obtained from all samples collected from all parts for each zone was calculated 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.

Results

Table 1 shows the chemical characteristics of the soil samples that were collected at the locations of plant sampling. There was no statistical difference in pH of the water extracts of soils, electric conductivity (EC), or cation exchange capacity (CEC) between zone 1 and zone 2. Evaluated pH and EC level revealed that the water extracts of the soils in both zones were mildly acidic and there was no problem with salinity; however, the soil samples associated with plants in zone 1 had slightly lower pH than zone 2. To better characterize nitrogen species levels, we measured total nitrogen and nitrate levels. Results indicated that both parameters were slightly lower in zone 1 than zone 2, although there was no statistically significant difference. Total contents of each metal (Cd, Co, Zn and Pb) in the soil samples of zone 1 were generally below the maximum allowable concentration of the USA.[36]

The ratio of total Cu in zone 1 compared to that in zone 2 was approximately 32 fold and approximately 61 fold greater for available Cu; levels that were higher than toxicity threshold levels.[38] However, the available Cu concentration for plants in zone 1 was 38% of total Cu level. There were no significant differences in the available levels of the studied metals between soils of the two zones except for Cu. The soils of zone 2 displayed no exceptionally high metal concentrations, 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 plant species collected from different sites. In general, plant species in zone 1 contained significantly higher Cu concentrations in studied tissues than those growing in zone 2 soils.

As we compared the levels of Cu between tissues in *Mimosaceace prosopis fracta*, we found a significant increase in copper level in roots, stems and leaves, respectively. The roots of *Mimosaceace prosopis fracta* accumulated Cu up to approximatley 5-fold of its level in leaves and to 2-fold its content in stems. The roots of *Mimosaceace prosopis fracta* grown in zone 1 accumulated most Cu compared with the tissues of other plant species. On the other hand, the copper levels in shoots (leaves and stems) of *Mimosaceace prosopis fracta* species was above the critical level for copper toxicity.[38] The ratio of Cu in roots of *Mimosaceace prosopis fracta* growing in zone 1 to that in the roots of the same plants in zone 2 was approximately 9. With regard to leaves organelle contents of Cu, the plant 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 *Mimosaceace prosopis fracta* was 7.92. The level of copper in chloroplasts of *C. ambrosioides* leaves was 2 times higher than vacuole Cu level.

The biomass characteristics of plant species and the leaves of chlorophyll content are shown in Table 3. In studied plants 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 an insignificant increase in dry weight of its roots. Leaf chlorophyll contents of this plant species collected from zone 1 were also significantly higher than those of zone 2. Table 4 shows antioxidative enzyme activities and the levels of oxidative damage biomarkers of different parts of the studied plant. The enzyme activities in each of the tissues were significantly higher than those of zone 2 and they were higher in leaves, stems and roots, respectively, in both zones. In addition, the leaves had significantly higher SOD and CAT activities than roots. On the other hand, there was only a significant increase in CAT activity in stems as compared with roots.

Both parameters, MDA and dityrosine, were insignificantly higher in tissues or studied plants that were grown in zone 1 as compared to the same plant species in zone 2. 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.

Discussion

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 contaminant originating from different human activities, including mining and smelting of copper containing ores. Mining activities generate a large amount of waste rock and tailings, which get deposited at the surface. Accordingly, Cu toxicity has important implication for the effects of copper mine ecosystems prone to Cu stress. In this work, the contaminated field of study was located in a copper mine. Accordingly, the soil analysis revealed normal levels of heavy metals (Pb, Co and Cd) and toxic levels of Cu in which the rate of available concentration of this metal was quite high (approximately 37%) for plant growth. This high Cu availability may be attributed to our soil pH characteristic. It has been confirmed that low levels of this parameter cause an increase in Cu solubility and its release from the soil phase leading to the increase in copper uptake by roots.[39] In accordance with our findings, soil analysis reported in the Cyprus Skouriotissa copper mine showed that there was mild acidity and copper content of up to 787 mg $(kg\ DW)^{-1}$.[40] In our zones of investigation, normal growth of our studied plant in metalliferous soils without any visual and conspicuous symptoms of Cu-toxicity implied that it was tolerant to toxic levels of Cu. The plant species was endemic in copper mines and naturally adapted to contaminated soils by developing tolerance mechanisms to metal stress. Most of these mechanisms have already been recognized, such as accumulation of metals and internal protective responses that vary among plants species and among different tissues.[41]

Based on our results, the studied plant could not be considered hyperaccumulators because the accumulation levels of copper in tissues were lower than 1,000 µg/gr, the prescribed threshold limit for hyperaccumulators.[42] Our finding was in agreement with the reports of Shu *et al.*[43] and Brun *et al.*[44] who illustrated that metal accumulation ability varies between species and is affected by their intrinsic characteristics. In their study, copper was accumulated differently in *Paspalum distichum* and *Cynodon dactylon*, metal tolerant plants, collected from the Lechang tailing copper mine in 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 reported in many other studies.[45-48] Furthermore, *Pistacia terebinthus* and *Cistus creticus* collected from the Skouriotissa copper mine accumulated a considerable amount of the absorbed copper in their roots, although *Bosea cypria* accumulated most copper in its leaves.[40] In agreement with these documents, our studied plant showed copper accumulation partitioning in which most copper was accumulated or bounded in roots with restricted translocation of copper toward shoots. On the other hand, many studies confirmed that when copper is in excess, it can promote and stimulate the generation of Fenton-type reactive oxygen species leading to an increase in antioxidative enzyme activities as a defense system.[18,49,50] This response to excess copper can vary among plant species and among different tissues.[18] Accordingly, the observed increase in each antioxidative enzyme activity in *Mimosaceace prosopis fracta* from a 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 this plant 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 leaf chlorophyll content of this plant associated with zone 1 were higher than the same plant growing in zone 2, although this difference was not statistically significant. However, there was no significant difference in MDA and dityrosine levels. In comparison, the roots of *Mimosaceace prosopis fracta* revealed a significant increase in MDA and dityrosine with respect to leaves that may be attribute to considerable low activities of antioxidative enzymes in roots. From our documents, many studies illustrated an inhibition effect of excess copper on antioxidative enzymes.[51,52] Because of a higher copper content in the roots of this plant, it would exert a toxic effect on antioxidant enzymes leading to a significant decrease in their activities with respect to leaves. On the basis of our findings and illustrated documents, we believe that antioxidative enzymes play a key role in the defense system against oxidative damage and in the tolerance of *Mimosaceace prosopis fracta* in a Cu-rich environment.

To conclude, this study showed that antioxidative enzyme responses to Cu-stress protected *Mimosaceace prosopis fracta* against oxidative damage and were involved in the plant's tolerance in a copper mine.

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