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Metal Contamination Effects on Sunflower (*Helianthus annuus* L.) Growth and Protein Expression in Leaves During Development

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Metal-ion contamination (Cd, Cu, Pb, and Zn) on sunflower (*Helianthus annuus* L.) growth and total leaf protein expression were studied in the present work. The height, mass production, and metal distribution (Ca, K, Fe, Mg, Na, and P) in all plant fractions (roots, stems, and leaves) were evaluated. Sunflowers plants contaminated with four metal ions decreases height and mass by 35% and 40%, respectively, compared to control. Significant differences of total protein composition were noted after SDS-PAGE separation. Sunflower proteomics were more affected when 500 mg L^{-1} of metal ion was added as contaminant of both zinc and mixed ions solution. In these cases, proteins having a molar mass of 14.5, 34.5, and 54.0 kDa were present at a lower level and alterations in enzymatic activities (SOD and GR) were found. Sunflowers plants contaminated with zinc and the mixed ions solution showed some degree of oxidative stress.

KEYWORDS: Sunflowers; proteins; metal stress; cadmium; copper; lead; zinc

1. INTRODUCTION

Plant mineral nutrition is fundamental for plant growth and development. Optimal plant growth is only achieved after controlling the level of essential minerals. Generally, the increase of inorganic elements in the environment occurs due to human activities (industrial, agricultural, mining, and waste disposal practices). Metal-ion contamination is a serious problem because it causes both stress and physiological constraints that affect the vigor and growth of plants, although different species show different responses to metal toxicity (*1*). The stress is due to formation of reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, and hydroxyl radicals that cause cellular damage in aerobic organisms. Plants have mechanisms to prevent oxidative stress. Many enzymes and others compounds avoid this damage by inhibiting or quenching free radicals and ROS (*2*).

Frequently, plant or tissue growth is used as the parameter for monitoring the effects of different stress conditions, assuming plant development to be deficient under such conditions. Plants stressed with metal ions show morphological and biochemical alterations at the cell and tissue levels as well as through decreases in their development. All these parameters are commonly used for stress monitoring (*3*).

The sunflower (*Helianthus annuus* L.) has the capability to accumulate high concentrations of metals in its tissues (mainly in shoots and roots) with reasonable tolerances. For this reason, it has been used for phytoremediation processes, which employ the use of metal-accumulating plants like sunflower for removing and recycling excessive metals from soil or water, promoting environmental cleanup (*4*−*6*).

It is important to mention that the literature clearly shows that most of the heavy metals accumulate in the root system with some being translocated to the upper parts of the plants (*2*, *7*, *8*). The distribution of metal ions varies considerably depending on the plant species and metal ions. It is also known that specific peptides termed phytochelatins can chelate metal ions and make them unavailable to the cell metabolism, with immobilization of such ions in the vacuole (*9*). There is extensive information in the literature about the accumulation of heavy metals in the roots, in a way far more extensive than the effect of these metals in the leaves. Furthermore, apart from a general effect on cell metabolism, metal ions can consequently alter the general protein composition (*10*, *11*).

Other investigations have evaluated the physiological changes related to antioxidant species production (*12*−*16*). However, investigations on sunflower proteome alterations caused by metal-ion contamination are essentially nonexistent. Moreover, to the best our knowledge, the association between changes in sunflower development and their proteomes has not been investigated.

In this way, the aim of this work was to evaluate sunflower growth under metal-ion stress conditions and study total protein expression in sunflower leaves. Comparative sunflower proteome analyses using one-dimensional (1-D) electrophoresis were performed on sunflowers treated with high concentrations

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of micronutrients (Cu and Zn) or trace elements (Cd and Pb). The effects of metal-ion contamination on the activities of three antioxidant enzymes (catalase, CAT; glutathione reductase, GR; and superoxide dismutase, SOD) in sunflower leaves were also evaluated.

2. MATERIAL AND METHODS

2.1. Reagents. All solutions were prepared with analytical reagent-grade chemicals purchased from Merck (Darmstadt, Germany), J. T. Baker (Phillipsburg, NJ) and Bioagency (São Paulo, SP, Brazil). Distilled−deionized water (18.2 MΩ cm) was purified through a Milli-Q water purification system (Millipore, Mosheim, French).

2.2. Plant Material and Growth Conditions. Sunflower seeds (*Helianthus annuus* L.) were germinated and grown in plastic pots (1 L) filled with 400 g of soil from Piracaia, Brazil, or with a mix of 320 g of the same soil with 80 g of vermicompost (humic material) from Campinas, Brazil. Details about the characterization of the vermicompost have been reported elsewhere (*17*).

The plants (one plant for each pot) were grown for 40 days (during autumn season) using seven different treatments at average temperatures of 18 and 24 °C (night and day, respectively) under ambient conditions. In the first treatment, sunflowers were grown in soil only. From the second to the seventh treatment, a mixture of soil and vermicompost was used. In the first two treatments, sunflower controls were irrigated with water. From the third to sixth treatment, sunflowers were irrigated with metal solutions (Cd(II), Cu(II), Pb(II), and Zn(II) at 500 mg L^{-1}), individually prepared from their respective nitrate salts. Finally, for the seventh treatment, a solution called "mixed ions solution" containing Cd(II), Cu(II), Pb(II), and Zn(II) was used. The concentration of each metal ion of this mixed ions solution was 500 mg L^{-1}. Irrigation, on alternate days, was done by adding 30 mL of the appropriate solution to each pot for a total of 600 mL during 40 days. Ten replicates were made for each treatment, except for mixed ions solution treatment, where a higher number of replicates ($n = 20$) was adopted. At the end of the experiment, a total of 70 sunflower plants were obtained.

2.3. Plant Sampling. After the 40 days growth, the sunflowers from each metal-ion treatment were harvested and cut into leaves, stems, and roots. The materials were rinsed three times with deionized water. One portion each of the fresh leaves, stems, and roots was used to analyze the protein composition. In this case, the washing process was performed as soon as possible to avoid protein degradation. Other portions of the plant materials were dried in an oven at 60 °C for 72 h before chemical analysis and biomass quantification.

2.4. Sample Preparation and Chemical Analysis. Approximately 200 mg of dry matter (root, stem, and leaves from each treatment) were decomposed using a microwave-assisted procedure with 6.0 mL of subboiling conc. HNO_3 and 0.5 mL of 30% (v/v) H_2O_2. The decomposition was performed in a laboratory DTG-100 microwave oven (Provecto Analitica, Jundiaí, Brazil) equipped with a temperature sensor, Teflon vessels, and a magnetron of 2450 ± 13 MHz with a nominal power of 1200 W. The microwave oven program was composed of four steps: 400 W at 5 min, 790 W at 8 min, 320 W at 4 min, and 0 W at 3 min. Afterward, the samples were heated (60 °C) to evaporate the excess HNO_3 and the volumes were finally adjusted with 2% (v/v) HNO_3 in 10 mL volumetric flasks for inorganic species analysis.

For Ca(II), K(I), Fe, Mg(II), Na(I), and P quantification, an inductively coupled plasma optical emission spectrometer (ICP OES), model Optima 3000DV (Perkin-Elmer, Shelton), was employed. A Perkin-Elmer model AAnalyst 600 (Norwalk) electrothermal atomic absorption spectrometer (ET AAS) with a Zeeman effect background correction system, furnished with a transversely heated graphite tube atomizer (THGA) and an AS-800 autosampler, was used to quantify Cd(II), Cu(II), Pb(II), and Zn(II). All measurements were made in triplicate. For checking the accuracy of the analytical method, a beech leaves (BCR 100) certified reference material was also used for Ca(II), K(I), Mg(II), and P determination.

2.5. Protein Extraction for SDS-PAGE Separation. The protein extraction procedure was based on that reported by Verbi et al. (*18*). Sunflower leaves collected from whole plant (1st to 10th leaf) were frozen in liquid nitrogen and manually milled in a mortar. The proteins were extracted by adding 1 g of sunflower leaves to a solution containing 370 μL of 1 mol L^{-1} Tris-HCl (pH 6.8), 600 μL of 10% (w/v) SDS, 300 μL of conc. glycerol, 150 μL of conc. β-mercaptoethanol, and 1580 μL of high-purity water. The sample was mixed with vortex for 20 min, and the remaining insoluble material was removed by centrifugation at 8500*g* for 5 min at 4 °C. When the extracts were not immediately used, they were frozen at −80 °C. The protein extracts from stem and root tissues were prepared by the same method as the leaf tissue.

The total protein content in all extracts was determined according to the Bradford method using bovine albumin as a standard (*19*). For total protein quantification, the dilution factor for stems and roots extracts was 25 and for leaves extracts 50.

A volume of 25 μL from the protein extract was used for SDS-PAGE separation. The separation was carried out with a vertical slab gel apparatus on a 135 mm × 135 mm × 1 mm gel from GE Healthcare Life Sciences (Piscataway). SDS-PAGE was done using a separation gel composed of 12.5% (w/v) acrylamide at pH 8.8 and 3.5% (w/v) stacking gel at pH 6.8 prepared according to Laemmli (*20*). Electrophoresis was run at 200 V and 30 mA until the bromophenol blue marker dye reached 1 cm from the bottom of the gel. The gel was stained with 1% (w/v) Coomassie brilliant blue G-250 for 2 h under gentle agitation.

For each electrophoretic separation, 25 μL of protein marker was added to a slot of the gel. The protein markers (MBI Fermentas, Hanover) included β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), restriction endonuclease *Bsp*981 (25.0 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).

The gel image was scanned, and the scan was analyzed using the Gel-Pro Analyzer program, version 3.1, to estimate the protein molar masses of samples through the protein markers. This program also allows the measurement of relative protein amounts. The mass of each band was calculated through a mass calibration curve constructed using the protein markers. The calibration curve enables establishing a relation between band volume and band mass.

2.6. Enzyme Extraction and Enzymatic Activity Evaluation. The enzyme extraction procedure was based on that reported by Vitória et al. (*7*) with minor modifications. Enzyme extraction and preparation were carried out at 4 °C. Frozen leaf tissues (3:1, buffer volume:fresh weight) were homogenized with a mortar and 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) containing 1 mmol L^{-1} EDTA, 3 mmol L^{-1} DTT, and 4% (w/v) polyvinylpyrrolidone. The homogenates were centrifuged at 10 000*g* for 30 min, and the supernatant was kept stored in separate aliquots at −80 °C prior to analyses.

The CAT and GR activities were determined as described by Ferreira et al. (*8*). CAT activity was spectrophotometrically assayed at 25 °C using a reaction mixture containing 1 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) and 25 μL of H_2O_2 (30% solution), which was prepared just before use. Twenty-five microliter samples were used to start the assay, and the activity was monitored by following the rate of H_2O_2 degradation at 240 nm for 1 min against a plant extract-free blank. GR activity was spectrophotometrically assayed at 30 °C in a mixture containing 1 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.5), 1 mmol L^{-1} 5,5-dithiobis(2-nitrobenzoic acid), 1 mmol L^{-1} oxidized glutathione, and 0.1 mmol L^{-1} NADPH. The reaction was initiated by addition of 50 μL of plant extract. The reduction rate of oxidized glutathione was followed by absorbance monitoring at 412 nm for 1 min.

PAGE was carried out under nondenaturing conditions in an 8% (w/v) polyacrylamide gel for SOD activity. A constant current of 15 mA was applied. The same amount of protein (25 μg) was loaded into each lane. Bovine liver SOD (Sigma) was applied to the gel as control. SOD activity was determined according to Gomes-Junior et al. (*21*) with minor modifications. The gel was rinsed three times with distilled−deionized water and incubated in the dark for 30 min. The reaction mixture consisted of 100 mmol L^{-1} potassium phosphate buffer (pH 7.8), 1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} nitroblue tetrazolium, 0.3% (v/v) TEMED, and 0.05 mmol L^{-1} riboflavin. Then, the gel was rinsed with distilled−deionized water and was exposed to light until develop-

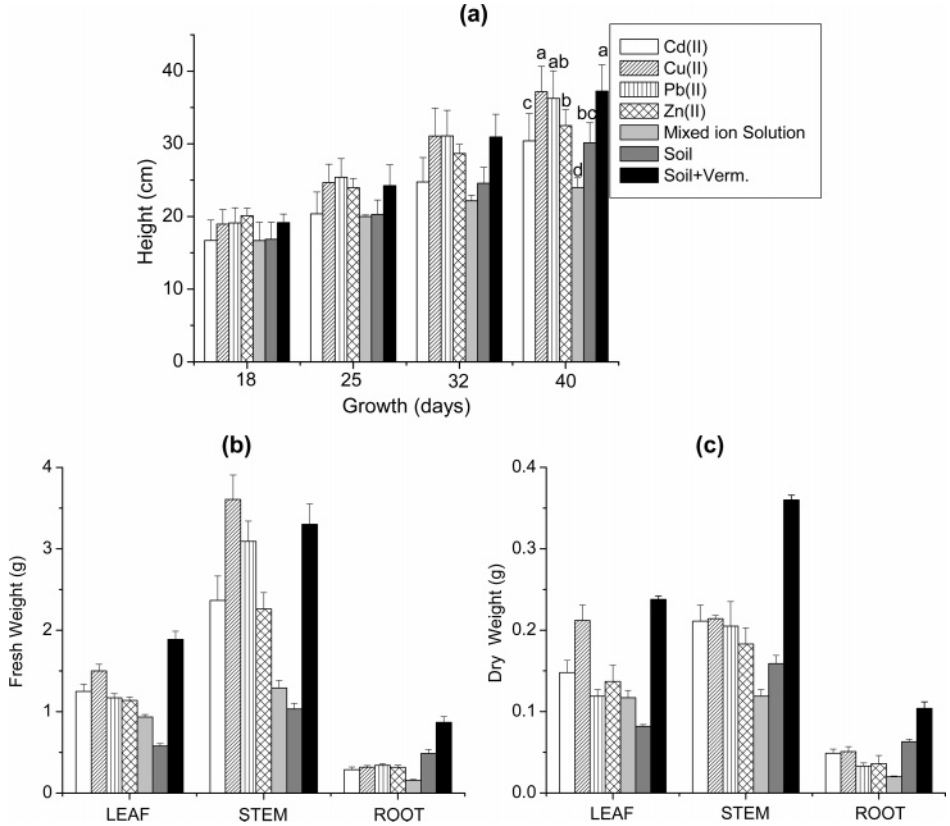

Figure 1. Sunflower development under different conditions: (a) growth for 40 days (average ± SD, $n = 10$); average values of each fraction followed by distinct letter indicate significant differences ($P < 0.05$) according to Tukey's multiple range test; (b) fresh mass obtained after harvest ($n = 5$) in each sunflower plant fraction; (c) dry mass ($n = 5$) obtained at 60 °C drying 72 h.

ment of colorless bands in a purple-stained gel. By adding 7% (v/v) acetic acid, the reaction was stopped.

2.7. Statistical Analysis. All data are presented as average values ± standard deviation (SD) obtained with at least three replicates. Differences among treatments were determined using ANOVA, taking $P < 0.05$ as significant, according to Tukey's multiple range test (*22*). The program ASSISTAT, version 7.3, available in the public domain, was used for statistical analyses (*23*).

3. RESULTS AND DISCUSSION

3.1. Contamination Effects of Metal Ions on Sunflowers. Figure 1a shows that sunflowers cultivated with soil plus vermicompost without additives reached the highest height (37 ± 4 cm). The smallest height (24 ± 1 cm) was obtained with sunflowers irrigated with the solution containing Cd(II), Cu(II), Pb(II), and Zn(II). Thus, the height of sunflowers in the presence of all metal ions decreased ca. 35% compared to those grown in soil plus vermicompost. Due to contamination with the four metal ions simultaneously (already indicated above), the highest mortality level (10 plants, 50%) was detected. This was the reason for the higher number of replicates in this treatment ($n = 20$) than with the others ($n = 10$) in order to always obtain 10 plants.

Contamination effects from metal ions (Cd(II), Pb(II), Zn(II), and mixed ions solution) were also observed with mass production. Plants cultivated in such conditions exhibited lesser masses than those cultivated with soil plus vermicompost (Figure 1b,c). This fact is more evident when the mixed ions solution was used. Under these conditions, the sunflower leaves and the whole plants were more fragile and with fewer roots (data not shown). The small amount of root tissue makes water and nutrient absorption more difficult as the absorption capacity of the root system is directly proportional to its mass.

Table 1. Ca(II), K(I), Mg(II), and P Determinations (mg g^{-1}, $n = 4$) with the Proposed Method (PM) and Using the BCR 100 Certified Reference Material (CRM)

analyte	PM	CRM
Ca(II)	4.70 ± 0.05	5.30 ± 0.05
K(I)	8.77 ± 0.25	9.94 ± 0.20
Mg(II)	0.733 ± 0.002	0.878 ± 0.017
P	1.23 ± 0.01	1.55 ± 0.04

Necrosis symptoms in the stem (near the root) were observed in sunflowers contaminated with Cd(II) and with the mixed ions solution (data not shown). Some plants contaminated with Pb(II) and Zn(II) also showed a twisted stem (data not shown). These facts demonstrate that the ions reached phytotoxic levels (*24*). Additionally, smaller masses were produced in plants cultivated in plain soil than those cultivated in the presence of vermicompost (Figure 1b,c). A 3-fold increase in leaf and stem mass production was verified as well as a 2-fold increase in root mass production (Figure 1b). This result can be considered as due to the mineral nutrients and high microbial activity of the vermicompost, as already reported by Atiyeh et al. (*25*), which contribute to plant development.

3.2. Nutritional Status. The accuracy of the method for determination of the metal content of the plants was evaluated using CRM material. The values obtained by ICP OES and the certified values are shown in Table 1. No statistical differences between both sets of results were found after applying the *t*-test at the 99% confidence level for K(I) concentration and 95% confidence level for Ca(II), Mg(II), and P concentrations (*22*).

Calcium is indispensable for maintaining the structure and normal operation of the cellular membranes. It was verified that this element was distributed throughout in the whole sunflower

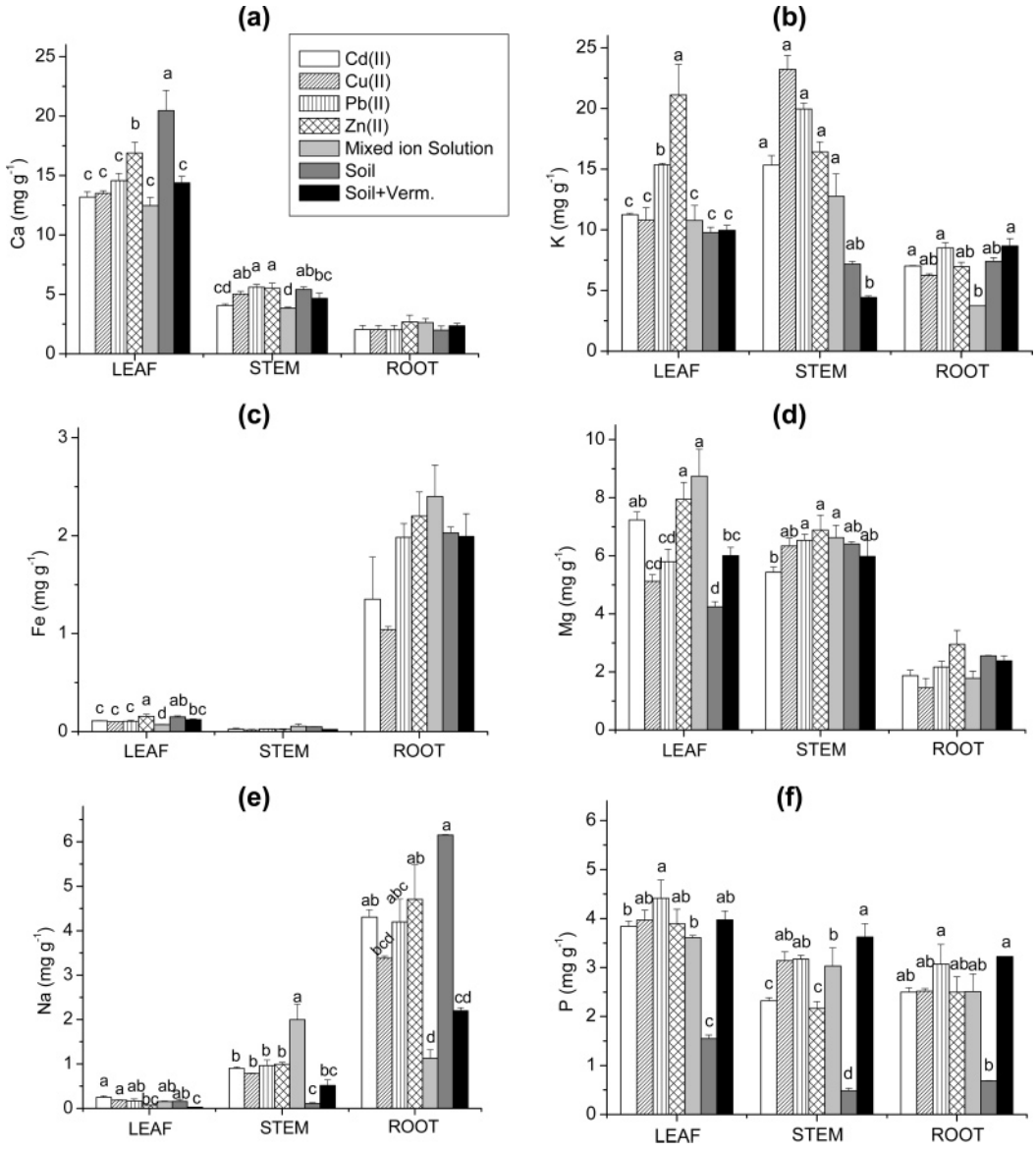

Figure 2. Nutrient concentrations in dry biomass (average ± SD, $n = 3$) from different plant fractions: (a) Ca, (b) K, (c) Fe, (d) Mg, (e) Na, and (f) P. Average values of each plant fraction followed by distinct letter indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. The absence of letters to a set of data indicates no significant difference ($P < 0.05$) according to ANOVA.

plant, although it is more concentrated in the leaves (Figure 2a) as the calcium ions are transported by the xylem in an ascending movement. Once located in the leaves, redistribution of Ca(II) to the plant is difficult (*26*). Sunflowers cultivated in soil showed the highest Ca(II) concentrations (Figure 2a). Due to ionic interactions of Ca(II) between the phenolic groups of humic acid present in vermicompost, its transport is more difficult in plants cultivated with this material (*27*).

The potassium ions (Figure 2b), which have a key biological role in activating enzyme and proteins synthesis, are also transported to the aerial parts of the plants by the xylem. In contrast to calcium, potassium distribution is facilitated due to its solubility (*26*), which can be visualized by comparing Figure 2a and 2b.

Iron can be absorbed in plants as Fe(II) and Fe(III), and it has a higher concentration in sunflower roots (see Figure 2c). This behavior is the same as that described by Madejón et al. (*6*), who noticed that other metal ions or the presence of vermicompost does not significantly alter iron absorption.

Magnesium transport (Figure 2d) is similar to that of calcium. However, more magnesium was transported to all sunflowers plant parts than in the presence of calcium (see Figure 2d and compare with Figure 2a). This fact is associated with the antagonistic effect of both ions wherein the presence of one of these ions decreases the absorption of the other (*26*) and explains the behavior of the Mg(II) concentration in leaves in the presence of Ca(II).

The largest accumulation of sodium was found in sunflower roots (Figure 2e), and its distribution is as follows: leaf < stem < root (*26*).

Phosphorus is an anionic macronutrient that carries out structural functions such as storage and energy supply for plants as well as protein synthesis, and its deficiency leads to lesser plant growth. Murillo et al. (*5*) and Madejón et al. (*6*) reported that the normal concentration of phosphorus in sunflower cultures is approximately 4 mg g^{-1}. As observed from Figure 2f, phosphorus was widely distributed in the sunflower, and its largest fixation was found with vermicompost fertilization. When the sunflower was cultivated in soil only, the appropriate level of phosphorus (4 mg g^{-1}) was not attained. These results demonstrate that its absorption was not hindered by the ions used as contaminants.

Table 2. Trace Element Determination (average ± SD, n = 3, dry matter) in Different Fractions of Sunflower Plants

		concentration (mg kg $^{-1}$)			
fraction	treatment	Cd(II)	Cu(II)	Pb(II)	Zn(II)
leaf	Cd(II)	147 ± 2	*a*	*a*	58 ± 8
	Cu(II)	*a*	19 ± 3	*a*	49 ± 4
	Pb(II)	*a*	5 ± 1	23 ± 0	49 ± 7
	Zn(II)	*a*	8 ± 0	*a*	673 ± 57
	mixed ions solution	379 ± 24	*a*	*a*	509 ± 46
	soil	*a*	24 ± 4	*a*	81 ± 17
	soil + verm.	*a*	9 ± 3	*a*	46 ± 8
stem	Cd(II)	426 ± 16	25 ± 10	*a*	43 ± 2
	Cu(II)	*a*	83 ± 6	*a*	77 ± 12
	Pb(II)	*a*	31 ± 1	35 ± 3	73 ± 7
	Zn(II)	*a*	8 ± 5	*a*	1365 ± 56
	mixed ions solution	1403 ± 313	125 ± 39	58 ± 19	3146 ± 304
	soil	*a*	61 ± 37	*a*	128 ± 9
	soil + verm.	*a*	*a*	*a*	74 ± 11
root	Cd(II)	1344 ± 130	*a*	*a*	*a*
	Cu(II)	a	1030 ± 97	*a*	*a*
	Pb(II)	a	a	1339 ± 231	a
	Zn(II)	a	a	a	2692 ± 342
	mixed ions solution	3321 ± 419	1257 ± 132	855 ± 93	5034 ± 756
	soil	a	a	a	26 ± 10
	soil + verm.	a	a	a	54 ± 16

[a] LOQ: Cd = 0.76 mg kg^{-1}, Cu = 0.48 mg kg^{-1} , Pb = 44.84 mg kg^{-1} , Zn = 7.56 mg kg^{-1}

Related to those elements used from the third to seventh treatment, it is important to point out that Cu(II) and Zn(II) are responsible for several cellular processes involved in plant growth. However, these elements can produce toxic effects in the plant tissues at high concentrations. Excess Cu(II) inhibits the growth of plants, mainly due to an abnormal operation of the root system (*4*). Generally, Cu(II) contamination in plants is lower when compared to Cd(II), Pb(II), and Zn(II). This fact may be explained because copper ions strongly interact with both organic matter and colloids in the soil, becoming difficult for copper to reach the plants, so that addition of Cu(II) to the sunflowers did not induce a larger absorption in the leaves. The copper concentrations in leaves from all treatments were within a 3−20 mg kg $^{-1}$ range (Table 2), which agrees with the literature (*5*).

Zinc absorption was more pronounced when it was added to the culture. Its concentration reached phytotoxic levels in leaves with both the Zn(II) and mixed ions solution treatments (Table 2) when the data are compared with those from the literature (500−1500 mg kg^{-1}) (*5*).

For cadmium, the phytotoxic level was attained in all plant fractions. It was observed that the presence of the other ions (Cu(II), Pb(II), and Zn(II)) in the mixed ions solution increased the absorption of cadmium (ca. 3-fold). In sunflower leaves, cadmium concentrations were 147 and 379 mg kg $^{-1}$ for plants receiving treatments with Cd(II) and with the mixed ions solution, respectively. These concentrations are at the phytotoxic level (5−700 mg kg^{-1}) (*28*). At these concentrations, Cd(II) inhibits photosynthesis processes, structural changes in the chloroplasts are observed, and the amount of chlorophyll is decreased (*29*, *30*). The literature reports a decrease of 40% in chlorophyll due to the presence of Cd(II) (*14*).

Absorption of Pb(II) was only detected in sunflower plants contaminated with this metal. Its phytotoxic level is considered relatively low when compared to other metal ions. Lead is basically restricted to roots (*5*), and the normal level of Pb(II) in leaves is 2−5 mg kg $^{-1}$. The lead concentration determined in sunflower leaves from plants contaminated with this element was 4-fold higher than the normal level.

Table 3. Total Protein Content in Each Fraction of the Sunflower (Fresh Mass) Determined by the Bradford Method (average ± SD, *n* = 3). Values in the Same Column Followed by the Same Letters Showed No Significant Differences ($P < 0.05$) According to Tukey's Multiple Range Test.

	concentration (mg g $^{-1}$)		
treatment	leaf	stem	root
Cd(II)	7.7 ± 0.2 cd	2.6 ± 0.1 b	0.87 ± 0.03 e
Cu(II)	7.7 ± 0.3 d	2.91 ± 0.05 b	1.37 ± 0.01 c
Pb(II)	7.9 ± 0.6 bcd	2.53 ± 0.02 b	0.94 ± 0.04 e
Zn(II)	8.9 ± 0.5 abcd	3.8 ± 0.2 a	1.17 ± 0.02 d
mixed ions solution	9.0 ± 0.4 ab	2.1 ± 0.2 c	1.69 ± 0.06 b
soil	7.9 ± 0.3 bcd	2.70 ± 0.06 b	2.14 ± 0.03 a
soil + verm.	9.7 ± 0.6 a	1.1 ± 0.1 d	0.91 ± 0.01 e

3.3. Protein Levels. The total protein concentrations of the entire sunflower plant are presented in Table 3. The sunflowers cultivated in soil and those contaminated with mixed ions solution synthesized the same amount of proteins (ca. 12.7 mg g^{-1}, Table 3). The plants contaminated with Cd(II), Cu(II), and Pb(II) had the same protein level as plants cultivated in vermicompost (ca. 11.0 mg g^{-1}). However, sunflowers contaminated with Zn(II) had the highest protein production (13.8 mg g^{-1}).

Proteins in leaves exhibited the highest concentration of all treatments (see Table 3), followed by stem and roots. The protein concentration of 7.7−9.7 mg g^{-1} in leaves is similar to that reported by Sairan et al. (*31*). In that work, a range of 5.0−9.0 mg g^{-1} was observed in different leaves (1st to 10th). In another work (*32*), ca. 22.0 mg g^{-1} of protein in sunflower leaves was observed after 30 days. However, in that case, sunflowers were cultivated using 10 kg of soil in each pot.

3.4. Changes in Protein Expression. Alterations in protein expression in sunflower contaminated with metal ions, which were not noticed by analyzing total protein concentrations using the Bradford method, were possible to observe only after SDS-PAGE separation of the proteins from the leaves.

The sunflower leaf proteins found in the present work can be associated to proteins of *Helianthus annuus* (common sunflower) available in the database (*33*) through the protein molar mass estimated using the Gel-Pro Analyzer program, version 3.1. The data related to protein molar mass and protein amount were found in sunflower leaves and are shown in Table 4.

Protein leaf composition presented different results (see Figure 3 and Table 4). It is noted that the intensities of the bands from plants cultivated with soil and vermicompost without contamination (control, Figure 3h) as well as the amount of protein mass (calculated as described in 2.5) was higher than those plants cultivated under metal ions contamination. The lower intensities predict that proteins of similar molar mass were expressed at lower amount (see also Table 4). The excess of metal ions caused oxidative stress in sunflowers and may explain the reduction of the protein levels. Probably, the metal ions provoke disorders in the sunflower metabolism as well as generate toxic species that cause degradation of proteins (*34*). However, the loss of staining (Figure 3) can also reflect the reduction on number of chloroplasts per unit of leaf tissue after metal treatment and consequently the amount of proteins belonging to these organelles.

The leaves from plants irrigated with Zn(II) (Figure 3e) and the mixed ions solution (Figure 3f) were more affected in terms of protein composition (see also Table 4). Bands 1 and 11 had reduced levels of protein content. In the sample proceeding from

Table 4. Estimated Protein Molar Mass (kDa) and Protein Amount (μg) of Sunflower Leaves ($n = 3$)

band	Cd(II)	Cu(II)	Pb(II)	Zn(II)	mixed ions solution	soil	soil + verm.
1	52.5 (9. 9 ± 0.4)[a]	53.0 (13.3 ± 0.8)	52.0 (11.0 ± 0.7)	52.0 (4.7 ± 0.3)	53.0 (5.1 ± 0.1)	54.0 (9.2 ± 0.2)	53.0 (17 ± 3)
2	45.5 (1.7 ± 0.2)	47.5 (1.6 ± 0.1)	47.0 (1.7 ± 0.2)	45.5 (1.7 ± 0.2)	46.0 (0.9 ± 0.1)	48.0 (1.4 ± 0.3)	46.0 (2.1 ± 0.2)
3	42.5 (4.2 ± 0.1)	43.5 (4.2 ± 0.1)	43.0 (4.0 ± 0.4)	42.0 (2.9 ± 0.3)	43.0 (3.7 ± 0.5)	43.0 (4.2 ± 0.3)	42.0 (6.0 ± 0.3)
4	38.5 (1.4 ± 0.1)	39.0 (2.6 ± 0.2)	38.5 (1.6 ± 0.2)	38.0 (1.4 ± 0.3)	39.0 (1.9 ± 0.1)	39.0 (0.6 ± 0.1)	38.0 (3.8 ± 0.1)
5	34.5 (2.2 ± 0.1)	34.5 (4.7 ± 0.4)	34.5 (3.3 ± 0.2)	b	35.0 (2.3 ± 0.4)	b	34.0 (5.0 ± 0.2)
6	31.5 (6.3 ± 0.2)	31.5 (5.9 ± 0.3)	31.5 (5.6 ± 0.7)	31.5 (4.4 ± 0.1)	31.5 (5.7 ± 0.3)	32.0 (4.3 ± 0.3)	31.0 (7.5 ± 0.8)
7	27.5 (8.2 ± 0.4)	28.0 (8.4 ± 0.2)	28.0 (4.8 ± 0.2)	27.5 (5.1 ± 0.7)	27.5 (7.1 ± 0.4)	28.0 (8.1 ± 0.3)	27.5 (8.5 ± 0.7)
8	24.5 (0.7 ± 0.1)	24.5 (1.8 ± 0.1)	24.5 (1.8 ± 0.2)	24.5 (0.6 ± 0.1)	24.5 (0.6 ± 0.1)	24.5 (0.8 ± 0.1)	24.5 (2.2 ± 0.2)
9	22.0 (3.2 ± 0.1)	22.5 (3.5 ± 0.3)	22.5 (2.9 ± 0.2)	22.0 (2.2 ± 0.2)	22.5 (2.7 ± 0.3)	22.5 (1.2 ± 0.1)	22.5 (5.0 ± 0.4)
10	17.5 (2.5 ± 0.3)	18.0 (5.8 ± 0.2)	18.0 (4.9 ± 0.3)	17.5 (3.2 ± 0.4)	18.0 (3.4 ± 0.2)	18.0 (3.0 ± 0.3)	17.5 (6.4 ± 0.6)
11	14.5 (5.9 ± 0.3)	14.0 (5.7 ± 0.4)	14.5 (3.7 ± 0.1)	14.5 (3.3 ± 0.2)	14.0 (4.8 ± 0.5)	14.0 (2.6 ± 0.2)	14.5 (9 ± 1)

[a] Protein amount estimated by Gel-Pro Analyzer program, version 3.1, according to the optical densitometry of each band. [b] Protein not detected by staining with Coomassie Brilliant Blue G-250.

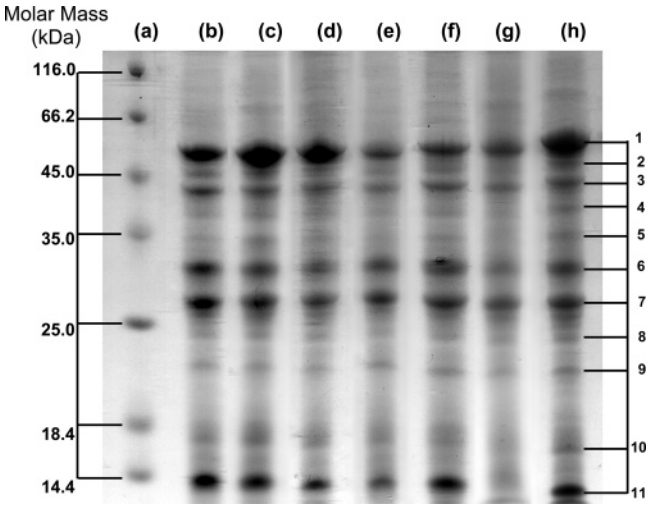

Figure 3. SDS-PAGE electrophoresis of protein extracts from sunflower leaves. The first lane in the gel (a) showed protein markers (molar mass ranging from 116.0 to 14.4 kDa). Other lanes showed sunflower leaf samples submitted at different treatments: (b) Cd(II), (c) Cu(II), (d) Pb(II), (e) Zn(II), (f) mixed ions solution, (g) soil, and (h) soil + vermicompost.

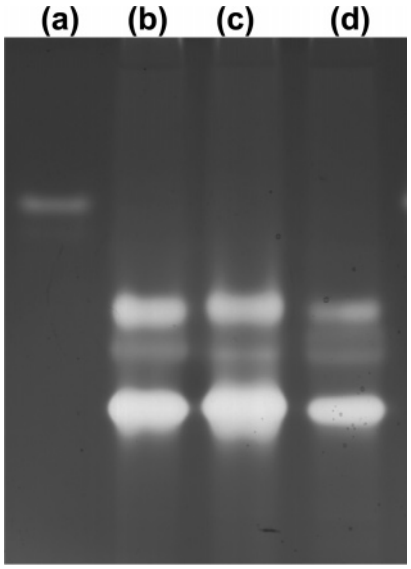

Figure 4. SOD activity staining following native PAGE separation of leaf extracts of sunflowers grown for 40 days: (a) SOD standard from bovine liver, (b) extract from soil + vermicompost treatment (control), (c) Zn(II) treatment, and (d) mixed ions solution treatment. Twenty-five micrograms was loaded onto each gel lane.

Zn(II) treatment (Figure 3e) at phytotoxic level (see Table 2), suppression of one band (number 5, 34.5 kDa) was also detected. The leaves from the mixed ions solution treatment also showed a phytotoxic level for cadmium but had no apparent influence on protein composition, and no significant alterations were detected when this metal ion was used separately as contaminant. The analysis of protein amount data (see Table 4) can corroborate this statement.

The prominent band (number 1, ca. 54 kDa, Figure 3) can be correlated by database analysis to the large chain of ribulose bisphosphate carboxylase (54.07 kDa). This protein, called rubisco, is present in leaf proteins, which participates in the Calvin cycle (fixation of CO_2) during the photosynthesis processes, so that the carboxylation ratio depends on the amount of this protein (*35*). Sunflower contamination by metal ions decreased the production of rubisco, mainly when irrigated with Zn(II) (Figure 3e) or the mixed ions solution (Figure 3f). Reductions from both treatments were ca. 70% when compared to the control (see Figure 3h and Table 4). Therefore, Zn(II) and mixed ions contamination induce a massive rubisco destruction and/or biosynthesis.

Proteins with a molar mass of 34.5 kDa (number 5, Figure 3) also had their expressions affected when sunflowers were grown in soil and irrigated with Zn(II) (Figure 3e). This molar mass, from the protein databank, could be for 1-aminocyclopropane-1-carboxylic acid oxidase (34.89 kDa), which participates in ethylene biosynthesis. Kasai et al. (*36*) also identified this enzyme through SDS-PAGE analysis. Production of ethylene is an important parameter because it can be used as a stress indicator. Hagemeyer and Breckle (*37*) comment that ethylene respost depends on metal ions concentration and the presence of interacting ions. In this work, reduction of 1-aminocyclopropane-1-carboxylic acid oxidase suggests a decrease on ethylene production.

3.5. Generation of ROS by Metal-Ions Contamination on Sunflower Plants. In order to corroborate those observations from Figure 3 and Table 4 and confirm ROS generation, some antioxidant enzymes were chosen. The choice for three antioxidant enzymes was made on the basis that oxidative stress will induce production of ROS, which can be dismutated by a series of reactions including enzymatic and nonenzymatic antioxidant systems. Among some of the key enzymes involved in these processes are SOD, CAT, and GR. Furthermore, these enzymes have been consistently shown to respond to a series of abiotic and biotic stresses, including oxidative stress induced by heavy metals. Therefore, analyses of the three enzymes were carried out to check the establishment of an oxidative stress condition. It is also known, as already mentioned, that there are several ways to check such aspects including analyses of compounds such as ascorbate, glutathione, carotenoids, lipid peroxydation status, H_2O_2, among others. Therefore, three key enzymes (SOD, which is the first enzyme in the line of defense

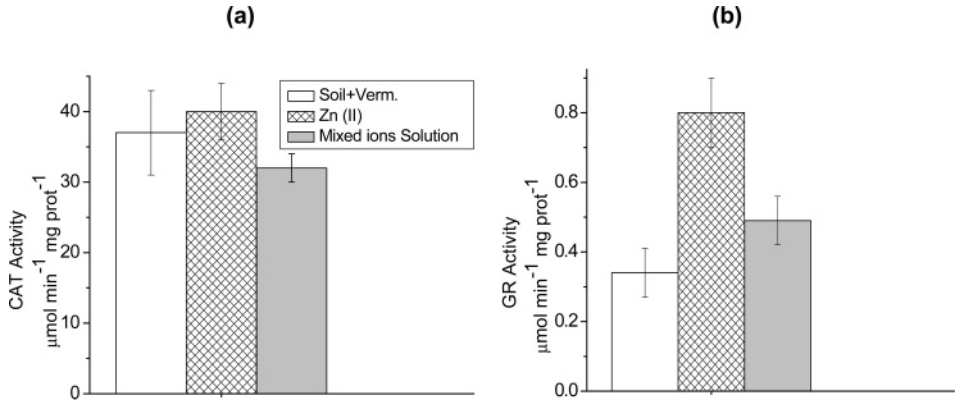

Figure 5. Enzymatic activities obtained in leaves extracts of sunflowers from different treatments after 40 days: (a) catalase (CAT) and (b) glutathione reductase (GR).

against ROS, CAT, which would be directly involved in the dismutation of the hydrogen peroxide formed particularly in the peroxysome, and GR, which has been shown to respond quite often to oxidative stress generated by heavy metals) were carried out (*2*). Although CAT and GR may be present as isoenzymes, the spectrophotometer assays are quite efficient, whereas the SOD activity staining gel is very efficient when compared to the spectrophotometer assay for this particular enzyme, which can be present as isoenzymes containing metal ions such as Cu−Zn, Fe, and Mn.

SOD activity exhibits variation that depends on the treatment used. In the zinc treatment (Figure 4c), the SOD activity was significantly higher than that of the control (Figure 4b), mainly considering the most electronegative isoenzymes band. However, after the mixed ions solution treatment (Figure 4d), an opposite behavior was observed. Despite these results, both indicate that metal-ion contamination provokes oxidative stress (*30*, *31*).

Figure 5a is related to the CAT activity. Similar stimulation responses between the treatments were observed. This fact suggests that CAT activity was not stimulated due to metal-ion contamination. The level of CAT activity found in leaves from soil and vermicompost treatment (control) is the same as that reported by Rios-Gonzalez et al. (*38*). However, GR activity (Figure 5b) was increased as a response to metal-ion exposure in order to promote a detoxification mechanism (*7*). The increase in GR activity indicates that this enzyme is maintaining the glutathione in its reduced form to be incorporated into phytochelatins (*21*) or for removal of hydrogen peroxide (*2*). Although total GR activity is increased, it is not possible to establish whether such increase is due to differential responses by distinct GR isoenzymes. It is important to mention the similarities on GR activity in the control when compared to those values reported by Gallego et al. (*12*). Enzymatic activity assays demonstrated that sunflowers adopted different ways to avoid the damage caused by ROS.

According to the results, it is concluded that metal-ion contamination significantly affected sunflower development, mainly when the mixed ions solution was used. In this case, whole plants showed less mass and smaller heights than the controls. Related to chemical species absorption, it was verified in the nutrition experiments that their distribution depends on the substrate employed. A high accumulation of metals (Cd, Cu, Fe, Na, Pb, and Zn) was observed in the root tissue of sunflowers. Similar behavior is found in the literature (*1*, *32*). Furthermore, metal-ion contamination induced deleterious effects on the protein content. The reduction in protein content observed due to excess metal ion is corroborated by those results already reported (*13*, *39*). The 1-D electrophoresis analysis allowed observing differences in sunflower leaf proteomes according to the different contamination conditions. Sunflower leaves contaminated with zinc showed more alterations in protein composition, although only slight variations in other parameters were noted. The sunflower leaf proteins within the molar mass range studied are metabolized to lower amounts under this condition. The present work indicates that protein and enzymatic analysis were helpful to demonstrate the generation of ROS on sunflowers plant stress by metal-ion contamination induced with mixed ions and in particular with Zn(II) by inducing degradation of various enzymes.

Finally, it is important to mention that 2-D electrophoresis can also be a good experimental tool to obtain the main proteins affected by metal-ions contamination, making characterization of such proteins possible after a well-established strategy on proteomics.

ABBREVIATIONS USED

ROS, reactive oxygen species; ICP OES, inductively coupled plasma optical emission spectrometry; ET AAS, electrothermal atomic absorption spectrometer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LOQ, limit of quantification.

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