

Evaluation of metal-ion stress in sunflower (*Helianthus annuus* L.) leaves through proteomic changes

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In this work, sunflowers (*Helianthus annuus* L.) were cultivated using soil and vermicompost as substrate, and plant irrigation was carried out using either a Zn solution or a mixed ions solution (Cd, Cu, Pb and Zn). After plant harvesting, the effects of metal-ion contamination on proteins expression (either up- or down-regulation) in sunflower leaves were evaluated using two-dimensional electrophoresis (2-DE), gel images and mass spectrometry (MALDI-QTOF MS). When Zn or mixed ions solution was added to the substrate, nine proteins showed different expressions. Another twenty-three protein spots also showed considerable variation when both treatments (Zn or mixed ions) were applied. Twelve of these proteins were successfully characterized, six of them being reported for the first time in *Helianthus annuus* L. Two other proteins showed new sequences that have been downloaded to the protein databank.

Introduction

Metal-ion contamination is a serious type of pollution in the environment. For plants, it can induce development problems such as growth decreases, reduced biomass production and other morphological and biochemical alterations.^{1,2} Metal sensitivities and toxicities in plants are influenced by the concentration, the type of metal and the stage of plant development. Information concerning metal homeostasis and plant tolerance are therefore important to elucidate the mechanisms affecting plant development. Generally, the mechanisms of metal-ion tolerance involve the exclusion of the metal-ion from uptake or translocation to the shoots, and immobilization of the metal in the cell wall to protect sensitive structures in the cytoplasm. This protection against metals occurs *via* a complex formation with organic acids and amino acids as well as by their binding to specific proteins. Metal toxicity can, however, inhibit protein activity or disrupt their structures.^{3,4}

Another metal detoxification mechanism in plants involves the activation of antioxidative enzymes systems such as catalase, peroxidase and superoxide dismutase. These enzymes are responsible for protecting the plant cells from the toxic effects of the reactive oxygen species that have their production increased during metal contamination and other types of stress.^{5,6}

Plant proteomics use many high-throughput biotechnological approaches to elucidate biological functions of plant proteins in

different environments including those resulting from exposure to metal-ion contamination, salinity, drought, air pollutants and extreme temperatures.⁷ Each condition produces a unique set of proteins in the organisms or a given tissue since protein activity, location and concentration are greatly dependent on environmental, physiological and pathological conditions.⁸ Plant stresses result in an increase of defense protein expression.⁷ Proteins related to antioxidative defensive mechanisms may, however, be either down- or up-regulated.^{7,9}

Among the proteins affected due to metal-ion contamination are the metalloproteins, which are responsible for various essential metabolic processes.² Metalloprotein evaluations also can contribute effectively to proteomic studies since they should give a new point of view for the understanding of the mechanisms of these metal-dependent proteins.¹⁰

As sunflower (*Helianthus annuus* L.) is considered the 5th most important culture in the world because of its oil production, has been employed in phytoremediation processes due to its capability to accumulate metals in its tissues,¹¹ only few studies in terms of oxidative stress have been carried out and few entries in the protein data bank related to this specie can be found, then the aim of this work was to evaluate systematically (*via* 2-DE, images and mass spectrometric techniques) the alterations in sunflower leaf proteomes under metal-ion (Cd, Cu, Pb and Zn) stress and to characterize some of the proteins affected by metal-ion stress. Additionally, investigations using the strategy adopted in this work on sunflower proteome alterations, and caused by metal-ion toxicity are almost unexplored.

Experimental

Plant material and growth

Sunflower seeds (*Helianthus annuus* L.) were germinated and grown under ambient conditions at temperatures ranging from

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18 to 24 °C (night and day, respectively). The seeds were cultivated in plastic pots (1 litre) with a mix of 320 g of soil from Piracaia, Brazil, and 80 g of vermicompost (humic material) from Campinas, Brazil. The plants (one plant per pot) were grown for 40 days using three different treatments. In the first one (control), sunflowers were irrigated with water only. In the second one, the sunflowers were irrigated with a solution containing $760 \mu\text{mol L}^{-1} \text{Zn}(\text{NO}_3)_2$. Finally, for the last treatment, a mixed-ion solution containing $440 \mu\text{mol L}^{-1} \text{Cd}(\text{NO}_3)_2$, $790 \mu\text{mol L}^{-1} \text{Cu}(\text{NO}_3)_2$, $240 \mu\text{mol L}^{-1} \text{Pb}(\text{NO}_3)_2$ and $760 \mu\text{mol L}^{-1} \text{Zn}(\text{NO}_3)_2$ was used. Irrigation, on alternate days, was done by adding 30 mL of the appropriate solution to the substrate contained in each pot, totalling 300 mg of each metal-ion after 40 days. The metal quantities added to the substrate were above the phytotoxicity level. At the end of the experiment, ten replicates were obtained for each treatment.

Protein extraction and two-dimensional gel electrophoresis

The protein extraction procedure was based on that reported by Garcia *et al.*¹¹ with minor modifications. For that, sunflower leaves were harvested, immediately frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$ until extraction. Ground leaves (1 g) were suspended into 3 mL of a solution containing 125 mmol L^{-1} Tris-HCl (pH 6.8), 1% (m/v) SDS, 1% (v/v) glycerol and 0.5% (v/v) β -mercaptoethanol. The homogenate was then centrifuged at $8500 g$ for 5 min at $4 \text{ }^\circ\text{C}$ and the pellet discarded. To precipitate proteins for sample cleaning, 5 mL of a precipitating agent (a solution comprising 20% (m/v) trichloroacetic acid and 0.2% m/v dithiothreitol–DTT in acetone) were added to 1 mL of protein extract. The pellets obtained were washed three times using cold acetone containing 0.2% (m/v) DTT. Then, protein concentration was determined according to the Bradford method using bovine serum albumin as standard.¹² For that, the protein pellets were re-dissolved employing a solution containing 1 mol L^{-1} Tris-HCl (pH 6.8).

2-DE was carried out following the manufacturer's (GE Healthcare, Uppsala, Sweden) recommendations.¹³ A mass of 2.4 mg protein was suspended into 300 μL of lysis buffer (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2% m/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.002% m/v bromophenol blue, 0.5% (v/v) carrier ampholytes (pH 3–10) and 1% m/v DTT) at room temperature ($25 \text{ }^\circ\text{C}$) for 5 min. Immobilized gradient pH strips (13 cm, pH 3–10, linear) were loaded with sample proteins during rehydration overnight at room temperature. Isoelectric focusing was carried out in a Multiphore II system (GE Healthcare), totalizing 16000 V h.

The second dimension separation was carried out at 25 mA per gel and 100 W during *ca.* 5.5 h in an Ettan DALT System (GE Healthcare) with lab cast 1 mm SDS polyacrylamide gels having a 12.5% (m/v) acrylamide concentration. After separation, the protein spots were visualized using 0.12% (m/v) colloidal Coomassie Brilliant Blue G-250.¹⁴ At least three gels of each treatment were obtained in independent experimental days.

The gels were scanned using an ImageScanner II (GE Healthcare) with the densitometer operating at 10000 dpi

resolution. ImageMaster 2D Platinum 6.0 software (GeneBio, Geneva, Switzerland) was used to analyze the gel images. Spot detections were done using default detection parameters and without manual editing. Spot volume was used instead of %volume (commonly employed to compare differential protein expression between gels) because the latter is more appropriate for gels with similar spot patterns.

Protein characterization by MALDI-QTOF MS

Protein spots that showed changes in expression due to different environmental conditions, as well as some others that were chosen randomly were characterized by MALDI-QTOF MS. For this task, an in-gel digestion of protein spots was performed using the Montage In-Gel Digest_{ZP} Kit (Millipore, Bedford, MA, USA) according to the manufacturer's recommendations.

All samples obtained by tryptic digestion were analyzed using the dried droplet method.¹⁵ The sample was acidified by adding a volume of 0.1% (v/v) trifluoroacetic acid (TFA) to the digested samples in a ratio 2 (TFA) to 1 (sample). The acidified sample (1 μL) was spotted on the MALDI plate for protein characterization, and kept at room temperature until complete solvent evaporation. MALDI matrix (1 μL) was then added to the sample, which also allowed drying at room temperature. This latter matrix was prepared from 1% (m/v) α -cyano-4-hydroxycinnamic acid, which was dissolved in a 1:1 (v/v) acetonitrile– H_2O solution containing 0.1% (v/v) TFA.¹⁶

MALDI-QTOF mass spectra were acquired in a MALDI-QTOF Premier mass spectrometer (Waters–Micromass, Manchester, UK). The mass spectra were obtained in the positive mode (LDI+) with a fixed nitrogen ion source and LockMass correction with 0.1% (v/v) phosphoric acid using the following main parameters: mass range from 880.0 to 3000.0 Da, peak detection threshold for MS/MS of 1500.0, mass threshold of 200.0 Da, scan time of 2 s, resolution of 10000 in “V” mode, trigger threshold of 700 mV, signal sensitivity of 80 mV, and microchannel-plate photomultiplier set to 2100 V. Each spectrum was collected over a 1 s scan, and the spectra were accumulated over *ca.* 2 min. The instrument was controlled by MassLynx 4.1v software. All mass spectra were processed into peak list files with a *.pkl extension using ProteinLynxGlobalServer 2.2.5v (Waters, UK).

Protein identification was achieved by searching a database using the peptide peak list (*.pkl file) masses and intensities files generated by MALDI-QTOF pos-processing mass spectra through ProteinLynxGlobalServer. Identification of proteins was performed using ProteinLynxGlobalServer 2.2.5v (Waters, UK). The Expressed Sequence Tag databank was obtained through The Institute of Genomic Research file transfer protocol download (ftp://ftp.tigr.org/pub/data/plantta/Helianthus_annuus)¹⁷ which was added and compiled into PLGS 2.2.5v to generate a concise open reading-frame of protein amino acids. Monoisotopic peak lists were processed with the following search parameters: HELIANTHUS-1.0 databank field input file, one missed cleavage, tryptic digestion, carbamidomethylation as a cysteine modification. The search error tolerance was set at 5 ppm with a $[\text{M} + \text{H}]^+$ charge state.

Results and discussion

Influence of metal-ion contamination in the protein profile

In an earlier work¹¹ we used one dimensional electrophoresis separations to evaluate the effect of metal-ion contamination on sunflower development as related to height, mass production, nutrition status, and protein level. Significant differences were observed for protein expression in sunflower leaves. Inspired by this result, we performed a series of new experiments using 2-DE and mass spectrometry to elucidate in more detail which sunflower proteins would be up- or down-regulated according to some specific metal-ion contamination; hence, those proteins of potential biotechnological interest for metal-ion detoxification and accumulation.

Using control sunflower leaves, more than 270 spots (Fig. 1A) were reproducibly detected in the 3–10 pH range ($n = 3$, 70% match). But when sunflower plants were irrigated with a zinc solution (Fig. 1B) or with a mixed ions solution (Fig. 1C) only *ca.* 105 ($n = 3$, 58% match) and 135 spots ($n = 3$, 55% match) were detected, respectively. Then, the presence of different metal-ions was found to result in considerable alterations of sunflower leaf protein compositions. Additionally, the comparison between proteomes was based on the expression of those more affected proteins so as to evaluate the sunflower plant development responses obtained after each metal treatment.

The proteins with the most remarkable differences in expression are identified in Fig. 1A. Representative examples of four different protein profiles are also highlighted in Fig. 2. The proteins depicted in Fig. 2 and some others also presented in the Fig. 1 were quantified (in terms of absolute volume). The absolute volume of the spot was determined using ImageMaster 2D Platinum 6.0 software (GeneBio, Geneva, Switzerland). This software calculates spot volume by multiplication of spot area (expressed in mm^2) and spot intensity (based on the highest calibrated pixel intensities in the spot from which the background has been withdrawn). The same procedure to proteins from contamination treatments was done. These volumes were then compared to those from the control (Table 1). Proteins were considered as up- or down-regulated when the spot volume changed at least 1.8-fold after contamination. This cutoff is the average value found in the literature concerning comparative studies on protein expression.^{18,19}

For the zinc and mixed ions treatments, 9 spots displayed significant changes in volume. For at least one treatment, 23 other protein spots also showed significant variation (see also Fig. 2 for some examples). The number of protein spots differentially regulated was more evident when the zinc solution was used for plant irrigation. Thirteen protein spot expressions were markedly decreased (1, 5, 7a–c, 9e and 11d, Table 1) and another 5 protein spots were up-regulated (9d and 15–18, Table 1). For the treatment with the mixed ions solution, 15 protein spots were down-regulated and 8 protein spots were up-regulated. Among the up-regulated proteins, 6 were detected only under this condition (7d, 8a–d and 9h, see Fig. 1C).

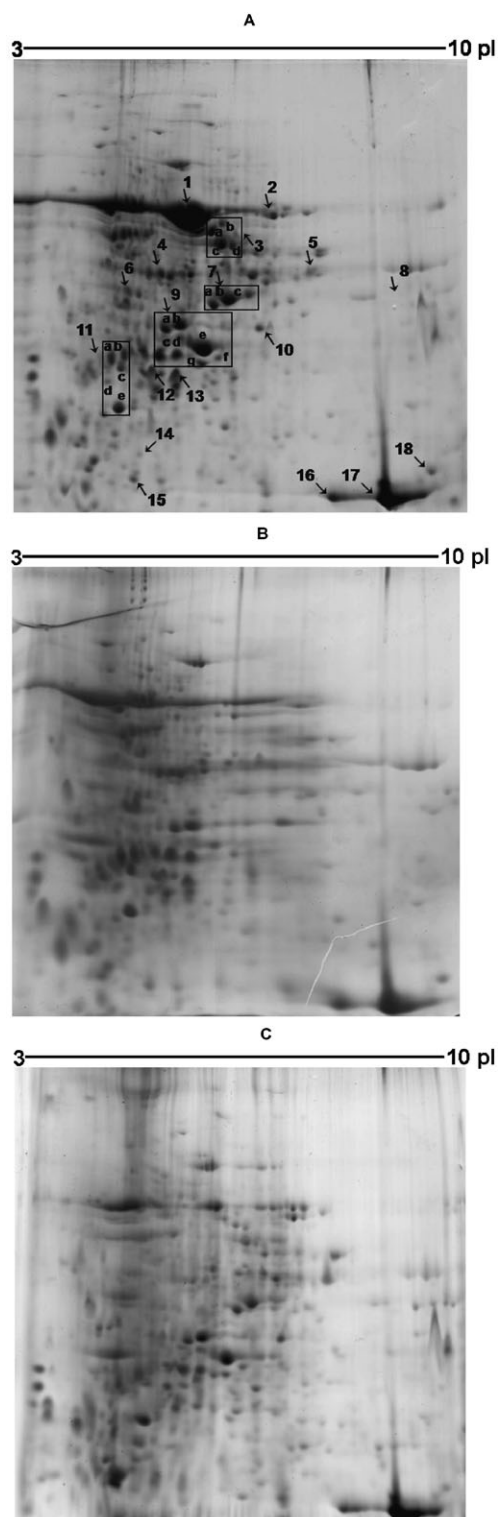


Fig. 1 Representative 2-DE gel for proteins for sunflower leaves. 2.4 mg of protein was loaded and run on 13 cm IPG strips (pH 3–10, linear). SDS-PAGE gels were used in the second dimension. The protein spots were visualized by Coomassie Brilliant Blue. (A) Control, (B) zinc contamination and (C) mixed-ion contamination. Arrows indicate the protein spots/regions analyzed, which were designated as numbers 1–18. The arrow 8 indicates proteins that were expressed only in the conditions specified in Fig. 1C.

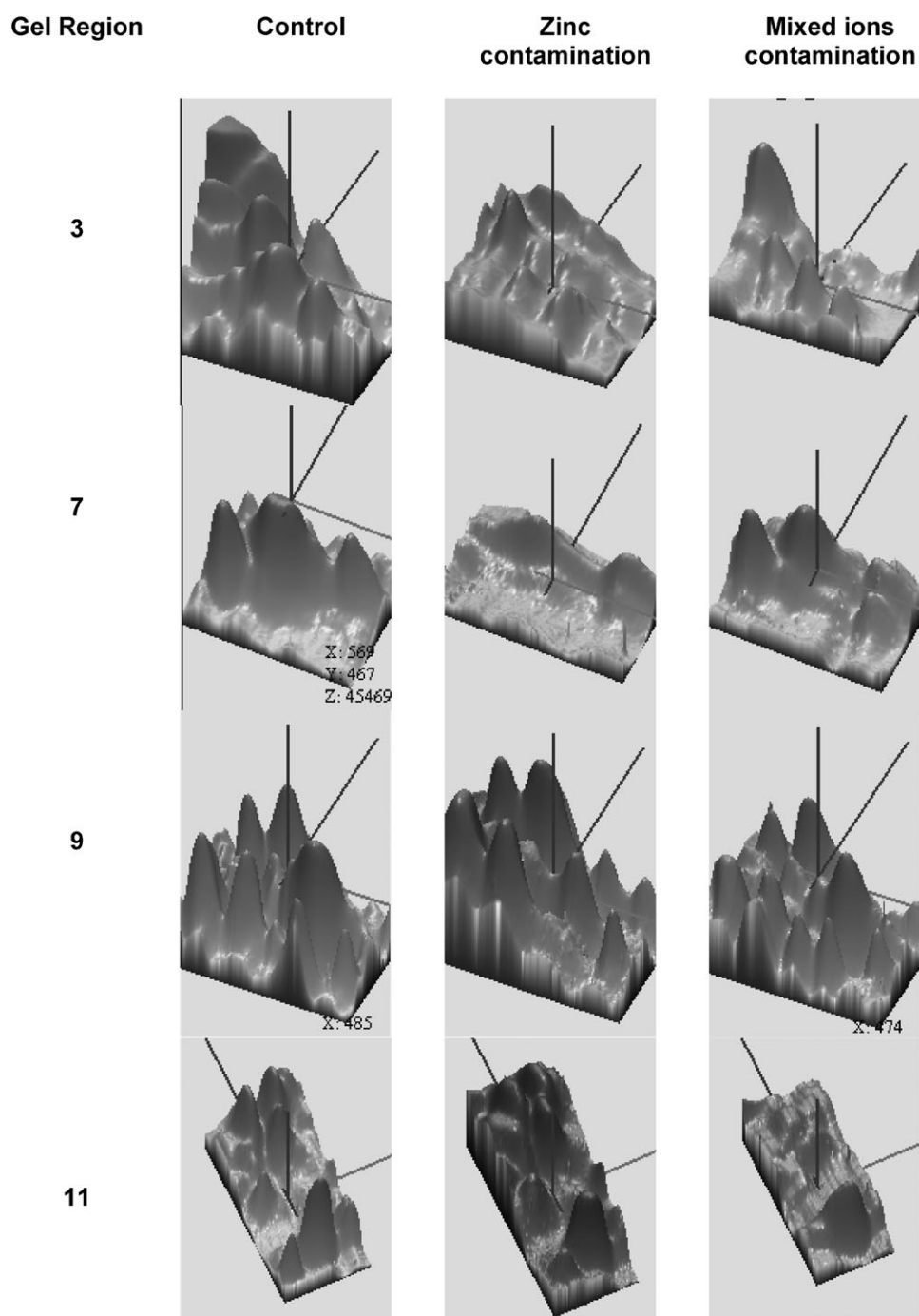


Fig. 2 Different gel regions were magnified using a 3D map to emphasize different expression of protein spots (numbered according to Fig. 1A).

These alterations in protein expression could be explained based on the zinc and cadmium phytotoxic levels found in sunflower leaves after growth: $Zn(II) > 500 \mu g g^{-1}$ in both treatments and $Cd > 5 \mu g g^{-1}$ in the mixed ions treatment. To obtain these results, leaves samples were decomposed using microwave-assisted procedure with nitric acid and the metal ions were quantified by electrothermal atomic absorption spectrometer (for more details, see ref. 11). Additionally, the presence of reactive oxygen species was verified in our earlier work¹¹ through enzymatic analyses. Changes on glutathione

reductase and superoxide dismutase activities were observed in sunflower leaves due to metal-ion contamination.¹¹

Characterization of proteins by MALDI-QTOF MS

Protein regulation as a response to metal-ion contamination is important to understand the role of the altered proteins in sunflower adaptation. Among 9 protein spots (that showed differences in expression when taken into account both metal treatments, see also Table 1), 3 of them (identified as 1, 5 and

Table 1 Comparative abundance (spot volume) of proteins in different conditions

Spot/ Gel Region	Volume (10 ⁴) ^a			Situation	
	Control	Zinc contamination (1)	Mixed ions contamination (2)	(1)	(2)
1	276.0	^b	19.6	Down	Down
2	13.0	11.7	8.5	Same	Same
3a	11.2	^b	3.7	Down	Down
3b	8.5	8.9	1.8	Same	Down
3c	24.5	^b	3.2	Down	Down
3d	12.4	^b	4.0	Down	Down
4	11.9	14.0	5.3	Same	Down
5	11.1	^b	23.7	Down	Up
6	8.0	8.9	^b	Same	Down
7a	16.5	^b	14.1	Down	Same
7b	55.0	^b	28.2	Down	Down
7c	18.7	8.9	19.8	Down	Same
7d	^b	^b	5.0	Same	Up
8a	^b	^b	4.2	Same	Up
8b	^b	^b	3.2	Same	Up
8c	^b	^b	4.5	Same	Up
8d	^b	^b	2.5	Same	Up
9a	14.6	17.4	4.4	Same	Down
9b	34.5	43.0	15.0	Same	Down
9c	21.0	31.6	15.9	Same	Same
9d	15.5	31.9	9.8	Up	Same
9e	63.1	23.2	36.6	Down	Down
9f	4.4	6.4	5.3	Same	Same
9g	9.5	^b	7.0	Down	Same
9h	^b	^b	8.7	Same	Up
10	6.5	7.1	15.5	Same	Up
11a	12.8	20.9	^b	Same	Same
11b	11.5	17.4	^b	Same	Down
11c	11.5	19.0	7.7	Same	Same
11d	5.2	1.1	^b	Down	Down
11e	18.1	26.5	26.5	Same	Same
12	11.9	6.8	13.3	Down	Same
13	18.2	28.6	9.6	Same	Down
14	3.2	^b	^b	Down	Down
15	5.6	16.5	9.1	Up	Same
16	42.5	89.9	27.3	Up	Same
17	94.1	236.4	98.4	Up	Same
18	8.7	20.2	6.9	Up	Same

^a Volume calculated using ImageMaster 2D Platinum 6.0 software.

^b Spot not determined using the ImageMaster 2D Platinum 6.0 software.

14—Fig. 1A) were successfully characterized by MALDI-QTOF MS. Some protein spots were also included for characterization to add new sunflower leaf information to the protein databank. Since the *Helianthus annuus* L. genome has not yet been completely sequenced, few proteins entries (1129) are available in SwissProt and TrEMBL, mainly when it is compared to *Arabidopsis thaliana* (51528) and *Oryza sativa* (142893), which already have their genome sequence elucidated. Despite this difficulty for *Helianthus annuus* L. protein identification, we succeeded in the characterization of 12 proteins (Table 2) and a good correlation (SCORE higher than 10.4 and COVERAGE between 69 to 88%) was obtained. Among the 12 proteins identified, six of them have been characterized for the first time for the *Helianthus annuus* L. species, whereas two other proteins showed new sequences that were also inserted in the protein databank.

Spot 1 was identified as ribulose biphosphate carboxylase large chain (the major leaf protein—called RuBisCO). This

Table 2 Proteins from sunflower leaves identified by MALDI-QTOF MS

Spot	Protein	Accession number
1	Ribulose biphosphate carboxylase large chain	AAB01594
2	Putative receptor protein kinase ^a	P85193
4	Cytochrome P450 ^a	P85191
5	Delta-12 oleate desaturase	AAL68982
6	Oxygen-evolving enhancer protein 1 ^a	P85194
9a	F6F9.12 protein ^a	P85200
9b	DEAD-box ATP-dependent RNA helicase 3 ^a	P85199
12	Unconventional myosin [Fragment]	AAB71528
13	Oxygen-evolving enhancer protein 2, chloroplast precursor ^a	P85189
14	Cell division protease ftsH homolog ^a	P85190
17	Hypothetical protein 1 ^a	P85192
18	CC-NBS-LRR-like protein [Fragment]	AAT08958

^a New sequences inserted in the ExPasy Proteomics Server.

protein participates in the Calvin cycle (CO₂ fixation) during photosynthesis processes as well as the oxidative fragmentation of the pentose substrate in the photorespiration process.¹¹

A significant down-regulation of ribulose biphosphate carboxylase was induced for both treatments (zinc and mixed ions), but it was more pronounced when the zinc solution alone was used for plant irrigation. Kim *et al.*²⁰ reported that other stresses such as salt, drought, high temperature and ozone contribute to the ribulose biphosphate carboxylase large chain accumulation. This protein was also up-regulated in *Arabidopsis thaliana* cells when they were exposed for 24 h to 200 μmol L⁻¹ CdCl₂.²¹ Tuomainen *et al.*²² reported, however, that cadmium contamination causes RuBisCO degradation in the *Thlaspi caerulescens* species (plant employed for bioremediation purposes).

Spot 5 was attributed to delta-12 oleate desaturase. This protein participates in oxidoreductase and phosphatidylcholine desaturase, and it can promote electron pair donors, resulting in molecular oxygen reduction and the production of two water molecules. The delta-12 oleate desaturase also participates in the fatty acid biosynthetic process.²³ Here, we found that changes in the expression of this protein is metal-ion dependent since down- and up- protein expression were observed when zinc and mixed ions solution were used, respectively.

The unconventional myosin [Fragment] was identified in spot 12, which has the molecular function of ATP binding and motor activity. The function of the CC-NBS-LRR-like protein [Fragment] (spot 18) is up to now unknown.²³ These last two proteins showed modification in their expression due to zinc stress. In this condition, unconventional myosin and CC-NBS-LRR-like proteins were down- and up-regulated, respectively.

Some proteins shown in Table 2 are being reported for the first time, hence their functions are putatively described based on homologies to others plants. Putative receptor protein kinase (spot 2) is responsible for ATP binding, protein binding, protein serine/threonine kinase activity and receptor activity as well as protein amino acid phosphorylation.²³ This protein abundance was not affected by metal-ion stress.

Cytochrome P450 (spot 4) participates in electron transport, promotes iron ion and heme binding as well as monooxygenase activity, which is responsible for the insertion of one atom of oxygen into an organic compound, whereas the other oxygen atoms are reduced to water.²³ This protein showed down-expression when the plants were irrigated with the mixed ions solution. According to Aina *et al.*,²⁴ cytochrome P450 expression was also inhibited in *Oryza sativa* L. roots due to cadmium toxicity.

Oxygen-evolving enhancer protein 1 and oxygen-evolving enhancer protein 2 (spots 6 and 13, respectively) were associated to the photosystem II complex. The first one stabilizes the manganese cluster, which is the primary site of water splitting. The second one may be involved in photosystem II regulation.²³ According to Tuomainen *et al.*,²² these proteins (involved in the energy metabolism) were up-regulated in *Thlaspi caerulescens* exposed to 500 mmol L⁻¹ ZnSO₄ for three weeks. However, we observed that these proteins were down-regulated due to the mixed ions treatment.

DEAD-box ATP-dependent RNA helicase 3 (present in spot 9b, see also Fig. 2), which is responsible for ATP-binding and hydrolysis and also shows helicase activity (catalyzes the unwinding of double-stranded nucleic acids). According to Roth *et al.*,²⁵ the exposure of *Arabidopsis thaliana* roots to cadmium contamination showed significant changes in terms of helicase family abundance (it was down-regulated). We observed similar behavior for mixed ions stress.

Cell division protease ftsH homolog (spot 14) seems to act as an ATP-dependent zinc metalloprotease and also to be related to metalloprotease and metal-binding.²⁴ This protein was not expressed when either zinc or the mixed ions solution were used for inducing stress.

Hypothetical protein 1 (spot 17) shows the function of peptidyl-prolyl *cis-trans* isomerase activity. There is no information about the F6F9.12 protein (spot 9a) function in the literature.²³

Other substantial changes were observed in protein expression (spots 5, 9a, 12 and 17, Table 1), which have not been previously reported. This contrast could be due to the different systems (cell cultures, roots, and leaves) or plant species as well as the different conditions investigated, making any comparative evaluation difficult.

Conclusions

The evaluation of metal-ion stress was successfully carried out in this work, inside the experimental domain. Metal-ion stress affected a significant number of proteins once that there was a decrease on the quantity of proteins in *ca.* 61% and 50% when the protein map from control sunflower leaves was compared with those obtained from plants irrigated with a zinc solution and with mixed ions solution, respectively. Additionally, metal-ion stress did affect a significant number of proteins, which showed up- or down-regulation in sunflower leaves. These alterations were more significant for those proteins related to energy metabolism (*e.g.* ribulose biphosphate carboxylase large chain, putative receptor protein kinase, oxygen-evolving enhancer protein 1, oxygen-evolving enhancer protein 2 and DEAD-box ATP-dependent RNA helicase 3).

In order to improve proteomic information related to sunflower, some proteins were also successfully identified. Eight new proteins sequences for *Helianthus annuus* L. were characterized. These proteins, reported for the first time, were appropriately inserted in the ExPasy Proteomics Server.

Finally, we consider that the combination of “omics” approaches would be expected to generate new information not only related to sunflower responses to metal-ion contamination but also related to other plants that show the capability of metal-ion accumulation, which could be used in the phytoremediation process.

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