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# Stress Responses of *Zea mays* to Cadmium and Mercury

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**Abstract**

A hydroponic experiment was carried out to characterize the oxidative stress responses of maize seedlings (*Zea mays* L. cv. Dekalb DK604) to cadmium (Cd) and mercury (Hg). Plants were grown hydroponically for 7 days in a nutrient solution supplemented with several concentrations of Cd and Hg: 0.0 (control), 6 or 30 $\mu$M. Growth was inhibited by both metals. The effect was more severe in plants exposed to Hg. Oxidative stress was caused by the exposure to the metals, as quantified by malondialdehyde and carbonyl accumulation, by-products of lipid peroxidation and protein oxidation, respectively. The activity of ascorbate peroxidase (APX) and superoxide dismutase (SOD), enzymes involved in the scavenging of reactive oxygen species, were measured upon metal treatment. We found an activation of a cytosolic APX isoform, as identified by using a specific polyclonal antiserum. However, there were negligible changes in SOD activity. Analysis of thiol-peptides revealed that at 6 $\mu$M Cd a remarkable increase in root reduced glutathione (GSH) content occurred, and little effect on the relative content of oxidised glutathione (GSSG) was observed. However, at 30 $\mu$M Cd and in plants exposed to 6 and 30 $\mu$M of Hg, GSH root content either remained stable or decreased significantly, while the proportion of GSSG increased. Moreover, only Cd was able to induce accumulation of phytochelatins at both assayed concentrations. Apparently, Hg was more toxic than Cd, as inferred from the magnitude of the changes found in the physiological parameters tested.

## Introduction

One of the major environmental problems caused by industrialisation is the increment in the concentration of heavy metals in the air, land and water (Nriagu, 1990). In particular, the area around mine facilities in Almadén (Spain) has the largest levels of environmental Hg in the world, due to the extraction and processing of Hg-mineral ore for centuries (Berzas et al., 2003). In opposition to other metals (i.e. Cu, Zn or Mn), Hg and Cd are not essential nutrients in most higher plants, and the exposure to relative low concentrations results in serious toxicity (Salt et al., 1995). One of the clearest phytotoxic symptoms induced by heavy metals is a diminution in plant growth, which is associated with disturbance of several metabolic processes, alteration of nutrient uptake and degeneration of cell ultrastructure (Hall, 2000). Besides, the appearance of oxidative stress has been well established (Schützendübel and Polle, 2002). It is thought that accumulation of reactive oxygen species (ROS)

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increases the cellular damages through oxidation of lipids (Sandalio et al., 2001), proteins (Romero-Puertas et al., 2002) and other macromolecules (Hall, 2000), leading to an oxidative burst. Thus, there is evidence of oxidative stress after exposure of several plants to Cd (Lozano-Rodríguez et al., 1997; Schützendübel et al., 2002) and to Hg (Cho and Park, 2000). Some of these stress responses have been attributed to changes in the activities of ROS scavenging enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APX; for a review see Sanita di Toppi and Gabbrielli, 1999). Depending on the organ sampled, the metal concentration and time of exposure, there were increases or losses in the activities of the referred enzymes, as summarized by Schützendübel and Polle (2002).

On the other hand, reduced glutathione (GSH) plays an important role in the defence against oxidative stress in plant cells, being involved in the complex enzymatic machinery that controls the intracellular levels of $H_2O_2$ (May et al., 1998). Changes in the amount of GSH compared with its oxidised form (GSSG) might be used as stress maker to assess acclimation of plants to their environment (Tausz et al., 2004). There are several reports showing changes in the levels of GSH and GSSG upon exposure of plants to different heavy metals (Schützendübel and Polle, 2002). A common initial response found in Cd-treated plants was a severe depletion of GSH tissue content (Dixit et al., 2001; Xiang and Oliver, 1998), which might recover after prolonged exposure (Schützendübel et al., 2001). This recovery might have been the result of increased GSH synthesis, probably through the over-expression of genes coding for enzymes of the glutathione synthetic pathway (Xiang and Oliver, 1998). Indeed, the importance of GSH to ameliorate Cd and Ni toxicity has been proved using transgenic plants overexpressing serine acetyltransferase (Freeman et al., 2004) or *o*-acetylserine(thiol)lyase (Domínguez-Solís et al., 2001) respectively, that resulted in elevated cellular levels of GSH compared to wild-type plants. Therefore, there is evidence pointing towards the implication, at least partially, of GSH in the tolerance to heavy metals. The depletion of the cellular pool of GSH in the presence of heavy metals, due to accumulation of GSSG and/or its polymerisation during phytochelatins (PCs) synthesis, may result in an increase in oxidative stress symptoms (Rauser, 1991; Xiang and Oliver, 1998).

A characterisation of the oxidative burst induced upon exposure to Hg in higher plants has been described in few reports (Cho and Park, 2000), and very scarce data are available about the content of GSH and GSSG. It is well known the affinity of $Hg^{2+}$ for thiol residues in proteins and peptides (Woolhouse, 1983), which might affect the levels of GSH/GSSG in plant tissues. The aim of the present work was to study physiological responses of maize seedlings to Hg, and compare them with those induced by Cd, a toxic element recurrently described in the literature. In particular, we considered very attractive to examine the relationship between oxidative stress and the depletion of GSH due to its oxidation to GSSG or the synthesis of PCs. According to our preliminary experience, these metals caused distinct phytotoxic effects in maize and pea plants (Lozano-Rodríguez et al., 1997; Hernández et al., 1998). Several oxidative stress indexes and ROS scavenging enzymes were evaluated, as well as changes in GSH, GSSG and other related non-protein thiols.

## Materials and methods

### *Plant material*

Maize (*Zea mays* cv. Dekalb DK604) seedlings were cultivated hydroponically as described in detail by Lozano-Rodríguez et al. (1997), using the following nutrient solution adjusted to pH 6.0: 2.0 mM $Ca(NO_3)_2$, 1.5 mM $KNO_3$, 1 mM $Mg(NO_3)_2$, 1.0 mM $KH_2PO_4$, 0.5 mM $MgSO_4$, 0.1 mM NaCl, 90 $\mu$M Fe-EDDHA, 23.5 $\mu$M $H_3BO_3$, 18 $\mu$M $MnSO_4$, 6 $\mu$M $CuSO_4$, 3 $\mu$M ZnSO4, and 2 $\mu$M $(NH_4)_6Mo_7O_{24}$. Seedlings were kept in a long-day photoperiod (200 $\mu$mol/$m^2$s; 16 h light/8 h darkness) at 25/18 °C, respectively. After 3 days of germination, selected seedlings grew for 5 days in control nutrient solution, and then were transferred to a nutrient solution supplied with Cd and Hg at three concentrations: 0.0 (control), 6 and 30 $\mu$M. Hypothetical speciation of both heavy metals in the nutrient solution was carried out by using Visual MINTEQ version 2.30 software with Lindsay's databases

(Gustafsson JP, Dept. of Land and Water Resources Engineering, KTH Stockolm, Sweden). The concentrations calculated for $Hg^{2+}$ were 6 and 30 $\mu$M, and for $Cd^{2+}$ were 5.4 and 27.0 $\mu$M. Shoots and roots of maize were collected after 7 days treatment, frozen in liquid $N_2$ and stored at −80 °C until analysis.

*Analysis of cadmium and mercury*

All samples were acid-digested prior to Cd and Hg analysis. Approximately 0.2 g of frozen ground tissue were placed in a boron–silicate chromatographic vial with 4 mL of capacity (capped with Teflon stoppers for digestion), and dried at 60 °C for 48 h. After determination of exact dry weight, samples were digested in autoclave (Presoclave-75, Selecta, Spain) for 30 min at 125 °C, $24.5 \times 10^4$ N $m^{-2}$, with an acid oxidative mixture $H_2O$:$HNO_3$:$H_2O_2$ (0.5:0.3:0.2, mL). The volume of the digests was adjusted to 10 mL following filtration. Cd concentration was measured by atomic absorption spectrometry with a hollow cathode Cd lamp using air–acetylene flame ionisation (Perkin Elmer 4000). Hg was also analysed by atomic absorption spectrophotometry using the same spectrophotometer with a hollow cathode Hg lamp, which was equipped with a cold vapour chamber and a $NaBH_4$ reduction reactor (Perkin Elmer MHS-20).

*Lipid peroxidation assay*

Lipid peroxidation was quantified as accumulation of the by-product malondialdehyde (MDA), according to Buege and Aust (1978). Prior to analysis, the frozen sample was ground in liquid $N_2$. 0.1 g of the powder was then homogenised in 1.0 mL of MDA reagent (15% w/v trichloroacetic acid, 0.37% w/v 2-thiobarbituric acid and 0.25 M HCl), and incubated at 90 °C for 30 min. The supernatant was clarified by centrifugation at 12,000 × *g* for 15 min, the absorbance read at 535 nm, and concentration calculated from the extinction coefficient of $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$.

*Protein oxidation*

Carbonyl content was measured after protein extraction as described by Romero-Puertas et al. (2002), with minor modifications. After incubation with 10 mM dinitrophenylhydrazyne, proteins were precipitated with 20% TCA, washed with 10% $H_2O$ in ethanol:ethyl acetate (1:1, v/v), and the pellet disrupted. The suspension was clarified by centrifugation at 12,000 × *g* for 5 min prior to absorbance determination at 370 nm (carbonyl concentration) and at 280 nm (total protein concentration).

*APX and SOD activities in non-denaturing gels and Western-blot*

After grinding the frozen samples in liquid $N_2$, 1 g of the powder was used to prepare an enzymatic extract with 2 mL of extraction buffer (30 mM MOPS, 5 mM Na-EDTA, 10 mM DTT, 10 mM ascorbate, 0.6% (w/v) PVP at pH 7.5), supplemented in fresh with 25 $\mu$L 0.1 M PMSF and 50 $\mu$L proteinase inhibitor cocktail (SIGMA, USA). To maintain the integrity of APX, special care was taken to add ascorbate in all extraction steps, keeping also all material at 4 °C. The homogenate was filtered and centrifuged (14,000 × *g* for 15 min at 4 °C), and the supernatant was stored at −80 °C in several single-use aliquots. Protein concentration of extracts was determined (Protein Assay; BioRad, USA) to load identical amounts of protein in native PAGE (10% and 12% acrylamide for APX and SOD, respectively).

For APX (EC 1.11.1.11) detection, 50 $\mu$g protein was separated by electrophoresis following essentially the protocol described by Jiménez et al. (1998). After incubation of gels with 4 mM ascorbate and 2 mM $H_2O_2$ in 50 mM Na-phosphate buffer at pH 7.0 for 20 min, the presence of APX was detected in the presence of 0.5 mM nitroblue tetrazolium (NBT) and 10 mM TEMED in 50 mM phosphate at pH 7.8. For SOD (EC 1.15.1.1) detection, a similar procedure was followed, although ascorbate was avoided in PAGE-native separation of proteins and subsequent incubations. SOD was detected following the procedure described by Beauchamp and Fridovich (1971), after incubating the gels with 1.25 mM NBT. $O_2^-$ was produced *in situ* by the photooxidation reaction of 50 $\mu$M riboflavine in the presence of 55 mM methionine in 50 mM Na-phosphate at pH 7.8.

To identify the bands in the native PAGE observed after *in gel* APX activity, we performed a Western-blot assay using a specific anti-cytosolic APX polyclonal antiserum (Dalton et al., 1996). Following APX staining, gels were incubated for 30 min in semi-dry electroblotting transfer buffer (50 mM Tris-HCl, 40 mM glycine, 1.5 mM SDS, 20% methanol, pH 8.4), and electrotransferred onto a nitrocellulose membrane (Pall, USA) using a Semi-dry Trans Blot SD (Bio Rad, USA), following standard procedures and under the conditions established by the manufacturer. The bonafide of the transfer was analysed by Ponceu's staining (0.2% Ponceau S in 1% acetic acid). The membrane was blocked with 1% BSA in TBS, and incubated overnight with the anti-cytosolic APX antibody (diluted 1/2000). A second incubation was performed with an alkaline phosphatase-conjugated secondary goat anti-rabbit antibody (Sigma, USA; diluted 1/2000), and the immunolabelled protein bands were revealed following standard protocols (Ausubel et al. 1987).

Images obtained were subjected to densitometric semi-quantification to determine changes in band intensity. Scanned images (ScanJet 3300C Hewlett Packard, USA) or pictures taken using a digital camera (Kodak 290, USA) were processed by using the Kodak 1D Image Analysis Software ver. 3.6. Regions of interest (ROIs) were of identical surface and pixel intensity was adjusted to the background. Data are given relative to the intensity of control samples.

### *Non-protein thiols analysis by HPLC*

Prior to extraction of non-protein thiols, the plant tissue was ground in liquid $N_2$. It is important to avoid prolonged storage of ground frozen samples at −80 °C, since we have observed alterations in the GSH pool by using spikes of thiols added to the samples. 100 mg of the frozen powder was thoroughly mixed with 300 $\mu$L of 0.25 N HCl, and a spike of N-acetyl cysteine was added as internal standard (50 $\mu$M final concentration). After centrifugation (12,000 × *g*, 15 min and at 4 °C), the clear extract was injected to a PLRP-S C18 polymer column (250 × 4.6 mm; Polymer Laboratories, U.K.), and eluted according to Meuwly et al. (1995), using the following gradient program (as for % solvent B): 2 min, 0%; 25 min, 25%; 26 min, 50%; 30 min, 50%; 35 min, 0%, 45 min, 0%; in an Alliance 2695 HPLC system (Waters, USA). Solvent A was 2:98 acetonitrile:$H_2O$ (v/v) plus 0.01% TFA, and solvent B 98:2 acetonitrile:$H_2O$ (v/v) plus 0.01% TFA. Detection was achieved after post-column derivatisation with Ellman's reagent, as described by Rauser (1991), and absorbance was measured at 412 nm. Non-protein thiols were quantified after integration of the peak area against that of the internal standard of N-acetyl cysteine. Identity of cysteine and GSH peaks was achieved by using commercially available standards.

The degree of GSSG was analysed by HPLC coupled to electrospray-mass spectrometry (HPLC-ESI/MS-TOF; Rellán-Álvarez et al., 2005). 100–500 mg of frozen tissue was homogenized in 200–1000 $\mu$L of chilled extraction solution (5% meta-phosphoric acid, 1 mM EDTA and 1% polyvinylpolypyrrolidone in 0.1% formic acid). After centrifugation (15,000 × g, 20 min at 4 °C) the supernatant was filtered through 0.22 $\mu$m polyvinylidene fluoride (PVDF) filters, and conserved at −80°C until further analysis. 20 $\mu$L of filtered sample were injected onto a Chromolith column (4.6 × 100 mm; Merck, Germany), and eluted (flux of 1 mL/min) with the following linear gradient program (as for % solvent B): 5 min, 10%; 6 min, 50%; 9 min, 50%; 11 min, 0%; 15 min, 0%, in an Alliance 2795 HPLC system (Waters, USA). Solvent A consisted of 0.1% formic acid in Milli-Q water and solvent B of 0.1% formic acid in acetonitrile. After ionisation with an Apollo electrospray ionisation source (ESI), GSH and GSSG ions were detected in a time of flight mass spectrometer (BioTOF II Bruker Daltonics, USA), operated at 2.8 kV of endplate and at 3.3 kV spray tip potentials in negative ion mode. Identification of peaks corresponding to GSH and GSSG was achieved using commercially available standards. Data of GSSG were calculated as a percentage of total glutathione (GSH + GSSG).

### *Statistics*

One-way analysis of variance was performed using the statistic software SPSS 11.0. Results were expressed as the mean ± S.D. unless noted

otherwise, and the value of $p < 0.05$ was considered significant.

## Results and discussion

### *Growth inhibition, analyses of oxidative stress indexes and heavy metal content*

Plants subjected to exposure to Cd and Hg suffered clear symptoms of phytotoxicity, as the fresh weight of shoot and root decreased significantly (Table 1). Similar diminution of organ growth was reported for Cd- (Guo et al., 2004; Sandalio et al., 2001; Schützendübel et al. 2001) and Hg-treated plants (Cho and Park, 2000). In parallel, Cd and Hg accumulated in plant organs at increasing levels concomitantly with the metal concentrations in the nutrient solution (Table 1). Higher proportion of metals was found in roots than in shoots. Thus, in plantlets exposed to metal concentrations of 6 and 30 $\mu$M the ratio of metal accumulated in roots to shoots was respectively: for Cd, about 35 and 35, and for Hg, 115 and 300. Therefore, little Cd and even less Hg were transferred to the aerial part of the plants. We also calculated the ratio of the metals that accumulated respectively in shoots and roots (Hg vs. Cd; Table 1). Interestingly, in roots Hg accumulated 2.3 and 3.8 times more than Cd, when supplied respectively at 6 and 30 $\mu$M. However, in shoots the ratio was 0.7 at both treatment concentrations. On the other hand, hypothetical speciation of Cd and Hg in the nutrient solution revealed that only $Cd^{2+}$ had a slightly lower concentration than expected: 5.4 $\mu$M instead of 6 $\mu$M, and 27.0 $\mu$M instead of 30 $\mu$M. This meant that the ratio of $Hg^{2+}$ to $Cd^{2+}$ concentration in the nutrient solution was 1.1. Taken together our data, lead to conclude that Hg accumulated in roots at a remarkably higher level than Cd, over the ratio of soluble cations concentration in the nutrient solution.

The inhibition of growth was accompanied by an increase in lipid peroxidation (MDA content) and protein oxidation (carbonyl content) as a result of an oxidative stress (Table 2). Similar responses have been described for MDA in maize (Lozano-Rodríguez et al., 1997), pea (Dixit et al., 2001; Sandalio et al., 2001), poplar (Schützendübel et al., 2002) and barley (Guo et al., 2004) under Cd exposure; and in tomato (Cho and Park, 2000) under Hg treatment. Some differences were observed with respect to the toxic effect caused and tissue susceptibility. In shoots, lipid peroxidation was clearly induced by both metals, but the effect of Cd was more pronounced than that for Hg. In roots, both metals caused a limited, yet significant rise of lipid peroxidation (Table 2). Protein oxidation was also enhanced by both Cd and Hg in roots and shoots. However, the effect of Hg was always larger than that of Cd. In particular, there was a remarkable carbonyl accumulation in plants exposed to 30 $\mu$M Hg, indicating an acute stress oxidation. The distinct action of each metal could be due to the different chemical properties and/or to the different rate of accumulation in plant tissues.

### *Ascorbate peroxidase and superoxide dismutase activities*

Several authors have highlighted the activation of ROS scavenging systems to ameliorate the

*Table 1.* Fresh weight (g/plant) and tissue concentration of heavy metals ($\mu$mol/g DW) in shoot and root from maize plants treated with Cd and Hg at the following concentrations ($\mu$M): 0.0 (control), 6 and 30 ($n = 5$) ± SD

| Treatment | Fresh weight (g/plant) | | Concentration of Cd ($\mu$mol/g DW) | | Concentration of Hg ($\mu$mol/g DW) | | Estimated Hg to Cd ratio | |
|---|---|---|---|---|---|---|---|---|
| | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root |
| Control | 0.83 ± 0.10 *a* | 0.47 ± 0.08 *a* | n.d* | n.d. | n.d. | n.d. | | |
| 6 $\mu$M Cd | 0.72 ± 0.08 *b* | 0.45 ± 0.05 *a* | 0.11 ± 0.01 *a* | 4.09 ± 1.20 *a* | | | | |
| 30 $\mu$M Cd | 0.47 ± 0.10 *c* | 0.35 ± 0.07 *b* | 0.43 ± 0.14 *b* | 23.45 ± 2.57 *b* | | | | |
| 6 $\mu$M Hg | 0.44 ± 0.08 *c* | 0.27 ± 0.04 *c* | | | 0.08 ± 0.03 *a* | 9.22 ± 0.17 *a* | 0.7 | 2.3 |
| 30 $\mu$M Hg | 0.38 ± 0.05 *c* | 0.24 ± 0.05 *c* | | | 0.29 ± 0.05 *b* | 89.65 ± 5.11 *b* | 0.7 | 3.8 |

*n.d. Not detected, below limit of sensitivity.
Values carrying different letters were significantly different at $p < 0.05$.

*Table 2*. Lipid peroxidation (nmol MDA/gFW) and protein oxidation (nmol carbonyl/mg total protein) in maize plants treated with Cd and Hg at the following concentrations ($\mu$M): 0.0 (control), 6 and 30 (n = 3) ± SD

| | Lipid peroxidation | | Protein oxidation | |
|---|---|---|---|---|
| | Shoot | Root | Shoot | Root |
| Control | 14.66 ± 1.12 *a* | 6.58 ± 0.41 *a* | 1.71 ± 0.54 *a* | 7.14 ± 0.85 *a* |
| 6 $\mu$M Cd | 32.60 ± 3.34 *c* | 10.98 ± 1.09 *b* | 3.78 ± 0.84 *b* | 9.21 ± 0.90 *b* |
| 30 $\mu$M Cd | 45.40 ± 7.36 *c* | 11.95 ± 0.61 *b* | 3.23 ± 0.28 *b* | 13.19 ± 0.85 *c* |
| 6 $\mu$M Hg | 19.26 ± 2.55 *b* | 9.72 ± 1.24 *b* | 4.04 ± 1.05 *b* | 10.58 ± 2.78 *b* |
| 30 $\mu$M Hg | 33.62 ± 4.94 *c* | 12.30 ± 0.28 *b* | 7.07 ± 1.01 *c* | 23.84 ± 4.85 *d* |

Values carrying different letters were significantly different at $p < 0.05$.

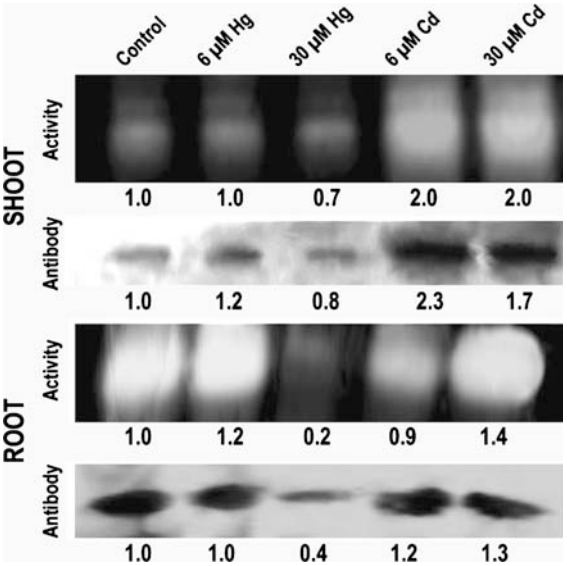


*Figure 1*. APX activity and Western-blotting of extracts prepared from shoot and root of maize treated with Cd and Hg at the following concentrations: 0 (control), 6 and 30 $\mu$M. A representative gel is shown out of least three replicates. Data underneath each band represent pixel intensity relative to control samples.

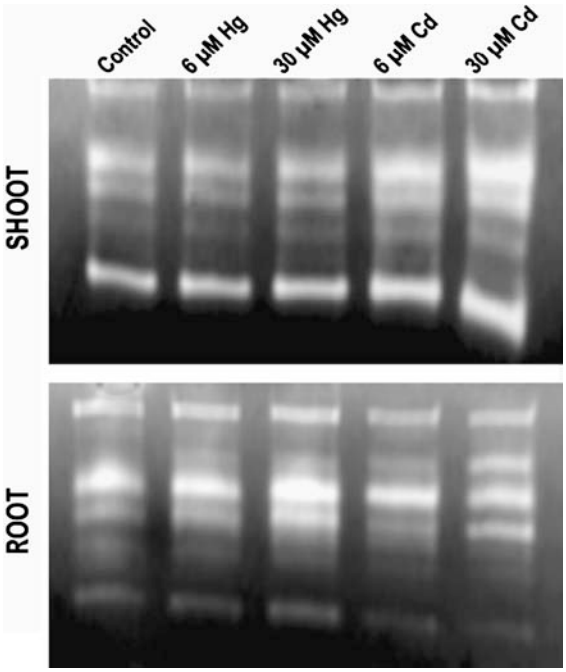


*Figure 2*. SOD activity of extracts prepared from shoot and root of maize treated with Cd and Hg at the following concentrations: 0 (control), 6 and 30 $\mu$M. A representative gel is shown out of least three replicates.

oxidative burst induced upon exposure of plants to heavy metals. APX, SOD, catalase, and other enzymes had been assessed under various metal concentrations (Schützendübel and Polle, 2002). In maize, by using an *in gel* approach, we analysed APX and SOD activities and found changes in response to increasing concentrations of Cd and Hg (Figures 1 and 2). APX staining resulted in a major colourless band. Western-blot analysis revealed that this major band corresponded to a cytosolic APX isoform, as we used a specific polyclonal antibody (Dalton et al., 1996). When plants were exposed to Cd, shoot APX activity increased, probably due to an increase in cytosolic APX, as visualised by Western-blot. However, APX activity decreased in the presence of 30 $\mu$M Hg, the decrease being related to a diminution of cytosolic APX protein (Figure 1). Higher activity was observed in roots than in shoots, but there was a sharp activity loss in the presence of 30 $\mu$M Hg. Again, that decrease in activity was concomitant with a diminution of APX cytosolic protein. This decline is in agreement with the high degree of protein oxidation observed in those samples (Table 2), and might

reflect an extreme toxic damage. High concentration of Cd (up to 50 $\mu$M) also caused depletion of several enzymes in pea leaf extracts, with a simultaneous increase of protein oxidation (Romero-Puertas et al., 2002). Indeed, a similar pattern in total peroxidase activity has been reported for maize (Lagriffoul et al., 1998), poplar (Schützendübel et al., 2002) and pea (Dixit et al., 2001) under Cd stress. It is conceivable that at mild stress conditions, plants respond by increasing APX activity, but under extreme toxicity a general failure of the metabolism causes its attenuation (Schützendübel et al., 2001, 2002). These effects could be also related to the amount of metals that accumulated differentially in the analysed tissues, at a much higher extent in roots than in shoots (Table 1).

On the other hand, SOD activity was not apparently affected by any of the metals, as no consistent effect related to the treatments with Cd or Hg appeared (Figure 2). The small activity changes were similar for all the enzyme isoforms present in each extract, indicating that those changes were due to protein loading, in spite of having tried to adjust protein concentration by using the Coomassie blue reagent. Several authors observed similar lack of response of SOD in Cd-treated Scots pine seedlings (Schützendübel et al., 2001). Besides, in leaves of pea plants exposed to concentrations of Cd over 40 $\mu$M only a diminution in SOD activity was reported, when plants suffered acute damage (Sandalio et al., 2001). In addition, Cho and Park (2000) found that SOD activity increased slightly in tomato seedlings exposed for 10 and 20 days to 10 and 50 $\mu$M Hg. Therefore, non-consistent changes in SOD activity might be observed upon exposure to Cd and Hg, as inferred from the data reported in the literature and our own results (Figure 2).

*Non-protein thiols concentration*

GSH and other non-protein thiols are known to be affected by the presence of several metals (Xiang and Oliver, 1998). Apart from being an essential metabolite in the cellular redox homeostasis (Noctor et al., 2002), GSH is the assembling block of phytochelatins (PCs), a family of cysteine-rich peptides that accumulate under metal exposure (Rauser, 1991). In the presence of Cd and Hg, the thiol-peptide pattern was altered (Figure 3). New thiol peaks appeared at longer retention time only in Cd-treated maize seedlings (Figure 3B). We have tentatively identified these peaks as corresponding to PCs, according to the retention time of the thiols accumulated upon Cd exposure in maize tissues using a similar chromatographic separation procedure (Meuwly et al., 1995). Moreover, maize plants subjected to prolonged treatment with 30 $\mu$M Cd (over 7 days) showed larger peaks of non-protein thiols in root extracts with identical chromatographic properties (data not shown), as it appears to be a typical response to Cd (Rauser, 1991). In

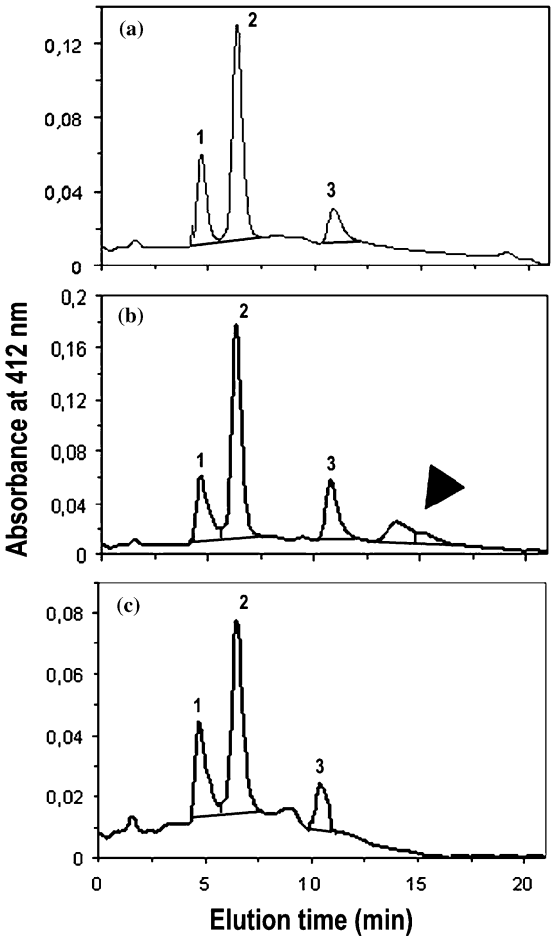


*Figure 3*. HPLC elution of thiol-peptides extracted from roots of maize treated with control (a), 30 $\mu$M Cd (b) and 30 $\mu$M Hg (c). 1. Cysteine; 2. GSH and 3. N-acetyl cysteine (internal standard). The solid black arrow indicates the appearance of putative Cd-induced phytochelatins, which were integrated as a unique peak.

*Table 3*. Thiol-peptide concentration (nmol N-acetyl cysteine/gFW) and percentage of GSSG vs. total content of GSH (%) in maize plants treated with Cd and Hg at the following concentrations ($\mu$M): 0.0 (control), 6 and 30 (n = 3) ± SD

| | Shoot | | | | Root | | | |
|---|---|---|---|---|---|---|---|---|
| | Cys | GSH | PCs | %GSSG | Cys | GSH | PCs | %GSSG |
| Control | 14.9 ± 3.9 *a* | 202.3 ± 27.0 *a* | n.d. | 29.5 ± 0.1 *a* | 47.3 ± 21.8 *a* | 206.8 ± 44.7 *a* | n.d. | 2.3 ± 0.3 *a* |
| 6 $\mu$M Cd | 18.7 ± 5.7 *a* | 186.1 ± 44.0 *a* | n.d. | 31.7 ± 1.5 *a* | 96.0 ± 39.2 *ab* | 385.2 ± 53.7 *b* | 12.7 ± 4.8 *a* | 2.5 ± 0.4 *a* |
| 30 $\mu$M Cd | 36.9 ± 5.3 *b* | 383.2 ± 16.6 *b* | 12.2 ± 0.7 *a* | 28.4 ± 0.4 *a* | 58.9 ± 36.0 *a* | 138.8 ± 48.2 *c* | 37.9 ± 6.7 *b* | 3.3 ± 0.7 *b* |
| 6 $\mu$M Hg | 14.4 ± 5.2 *a* | 155.1 ± 8.8 *c* | 7.6 ± 4.4 *a* | 27.5 ± 2.3 *a* | 78.9 ± 41.2 *ab* | 205.7 ± 10.0 *a* | < 2.5 | 4.9 ± 0.8 *c* |
| 30 $\mu$M Hg | 19.2 ± 6.8 *a* | 243.3 ± 46.1 *a* | < 2.5 | 24.4 ± 7.2 *a* | 188.9 ± 26.6 *c* | 149.5 ± 25.0 *c* | n.d. | 7.6 ± 1.4 *d* |

*n.d.: not detected.
Values carrying different letters were significantly different at $p < 0.05$.

addition, lower amount of Cd-induced thiols was observed in shoot extract only at the highest concentration of Cd (Table 3). Under Hg stress, we could not detect peaks corresponding to PCs (Figure 3C). Only in the shoot of plants exposed to 6 $\mu$M Hg, we could detect a minor peak at the same elution time as shown for Cd-treated maize (Table 3). Our findings agree with the known poor capability of Hg as a PCs synthesis inductor, as has been observed *in vitro* with recombinant phytochelatin synthase (Ha et al., 1999) or *in vivo* (Maitani et al., 1996). Therefore, it is possible the PCs might play a side role in Hg detoxification in maize seedlings.

On the other hand, GSH concentration varied depending on the metal concentration and the tissue analysed. In the shoot, GSH concentration augmented only at the highest concentration of metal supplied, being Cd a stronger inductor than Hg (Table 3). However, this effect was not accompanied with any significant change in the percentage of GSSG. A similar increment in GSH content was only found in roots of maize treated with 6 $\mu$M Cd, while in plants exposed to 6 $\mu$M Hg there were no changes. Plants grown in 30 $\mu$M of Hg or Cd showed a significant diminution of GSH in roots, which was concomitant with an increase in the percentage of GSSG (Table 3). Apparently, there was a rise under mild toxicity, but it declined when plants suffered acute stress (i.e. in roots of plants given 30 $\mu$M). These effects were also accompanied by an increase in the content of cysteine only in the root of plants exposed to 30 $\mu$M Hg (Table 3), tissue that showed low concentration of GSH and high GSSG percentage. Our results suggest that GSH might have been accumulated in mild stressed plants, according to the reported increases to levels above those of controls, which is common in prolonged Cd-exposure (Schützendübel et al., 2001; Vöeli-Lange and Wagner, 1996; Xiang and Oliver, 1998). However, above a certain toxicity threshold the metabolism is unable to attain the putative demand of GSH to keep redox homeostasis under control. In fact, when plants showed higher oxidative stress symptoms in the root upon 30 $\mu$M Hg treatment, the proportion of GSSG in root was almost three times that of control plants (Table 3). These results indicate that redox GSH/GSSG homeostasis was only compromised in tissues and organs that were severely affected, in agreement with Xiang and Oliver (1998).

## Conclusions

Hg showed a stronger toxic effect than Cd in the root of maize seedlings, as inferred from the found higher proportion of GSSG, enhanced carbonyl content and the negative effects on growth. Similarly, it was also observed that accumulation and activity of the cytosolic APX was severely affected in plants exposed to 30 $\mu$M Hg. Under this acute stress conditions, the pool of GSH was reduced, clearly indicating that cellular homeostasis might have been compromised. Only under mild stress, plants could probably acclimate and adjust its metabolism accordingly. It is possible that the higher phytotoxicity caused by Hg might be partially associated to its higher accumulation in the root. In future work, it is important to establish the thresholds of toxicity, either by lowering the metal doses or reducing the exposure

times. Characterisation of the cellular components interacting with the metals might also help to determine different mechanisms underlying metal toxicity and to understand the way plants can cope with those stress conditions.

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