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Polyamines and heavy metal stress: the antioxidant behavior of spermine in cadmium- and copper-treated wheat leaves

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Abstract

Polyamine metabolism, as well as spermine (Spm) antioxidant properties, were studied in wheat leaves under Cd^{2+} or Cu^{2+} stress. The oxidative damage produced by both metals was evidenced by an increased of thiobarbituric acid reactive substances (TBARS) and a significant decrease in glutathione under both metal treatments. Ascorbate peroxidase (APOX) and glutathione reductase (GR) activities were reduced by both metals to values ranging from 30% to 64% of the control values. Conversely, copper produced a raise in superoxide dismutase activity. The high putrescine (Put) content detected under Cd^{2+} stress (282% over the control) was induced by the increased activity of both enzymes involved in Put biosynthesis, arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). However, only ODC activity was increased in wheat leaves subjected to Cu^{2+} stress, leading to a lower Put rise (89% over the controls). Spermidine (Spd) content was not affected by metal treatments, while Spm was significantly reduced. Pretreatment with Spm completely reverted the metals-induced TBARS increase whereas metals-dependent H_2O_2 deposition on leaf segments (revealed using diaminobenzidine), was considerably reduced in Spm pretreated leaf segments. This polyamine failed to reverse the depletion in APOX activity and glutathione (GSH) content produced by Cd^{2+} and Cu^{2+}, although it showed an efficient antioxidant behavior in the restoration of GR activity to control values. These results suggest that Spm could be exerting a certain antioxidant function by protecting the tissues from the metals-induced oxidative damage, though this effect was not enough to completely avoid Cd^{2+} and Cu^{2+} effect on certain antioxidant enzymes, though the precise mechanism of protection still needs to be elucidated.

Abbreviations: ADC – Arginine decarboxylase; APOX – Ascorbate peroxidase; DAB – Diaminobenzidine; DAO – Diamine oxidase; GPOX – Guaiacol peroxidase; GR – Glutathione reductase; ODC – Ornithine decarboxylase; Pas – Polyamines; Put – Putrescine; SOD – Superoxide dismutase; Spd – Spermidine; Spm – Spermine; TBARS – Thiobarbituric acid reactive substances.

Introduction

Heavy metals are important environmental pollutants present in soils, and toxic levels of some of them (cadmium, copper, lead, etc.) could appear in natural and agricultural areas as a result of anthropogenic activity. Their presence in the atmosphere, soil and water, even in trace concentrations, can cause serious problems to all organisms (Benavides *et al.* 2005; Gratão *et al.* 2005). Cadmium is a non-essential heavy metal released into the environment by metal-working industries, heating systems, urban traffic or cement factories (Sanità di Toppi & Gabrielli 1999; Baycu *et al.*

2006). It is toxic to living cells at very low concentrations and since Cd is fairly immobile, its accumulation in soils can become strongly phytotoxic, producing growth inhibition and even plant death. This metal produces changes in membrane lipids (Ouariti *et al*. 1997) and affects enzyme activities (Van Assche & Clijsters 1990). Copper is widely distributed in nature and is an essential element for normal plant growth since it is constituent of many enzymes and proteins. However, it is toxic for plants at high concentrations, since it can produce lipid peroxidation through the formation of free radicals that disturb membrane permeability (Lovaas 1997).

Heavy metals are implicated in the generation of oxidative stress in plant cells. There is evidence that in pea plants exposed to Cd^{2+}, the antioxidant system might play a role in detoxification mechanisms (Dixit *et al*. 2001). Plants posses an antioxidative system to protect themselves against the damage produced by oxygen-derived radicals. This system is composed of antioxidant enzymes: ascorbate peroxidase (APOX), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and non-enzymatic compounds (ascorbic acid, glutathione, carotenoids, α-tocopherol) (Gratão *et al*. 2005).

Polyamines (Pas) are nitrogen compounds present in all living cells. The three main Pas, putrescine (Put), spermidine (Spd) and spermine (Spm), participate in different cellular processes ranging from growth promotion and cell division to inhibition of ethylene production and senescence (Tiburcio *et al*. 1997; Bagni & Tassoni 2001). Many papers report changes in Pas levels in relation to several types of environmental stresses, e.g. UV radiation (Kramer *et al*. 1991), osmotic and salt stress (Aziz *et al*. 1998) and heavy metal stress (Weinstein *et al*. 1986; Lin & Kao 1999). Two alternative ways for Put synthesis exist in plants: from arginine decarboxylation by arginine decarboxylase (ADC) and from ornithine decarboxylation by ornithine decarboxylase (ODC). ADC activity is usually reported to increase in response to stress whereas ODC activity is modify during growth and development (Bouchereau *et al*. 1999). In the last years, many authors have evaluated the possible antioxidant effect of Pas through the inhibition of lipid peroxidation (Lovaas 1997). It is suggested that they achieve this effect by binding to negative charges of membrane phospholipids thereby stabilizing membranes or by acting as radical scavengers.

The aim of this work was to study Pas and heavy metal stress, especially the antioxidant behavior of Spm against Cd^{2+} or Cu^{2+}-induced oxidative damage in excised wheat leaves.

Materials and methods

Chemicals

NADPH, GSH, GSSG, DTNB, GR, Put, Spm, Spd, and 1,6 hexanediamine were from Sigma Chemical Company (Saint Louis, MO). All chemicals were of analytical grade.

Plant material and treatments

Wheat seeds (*Triticum aestivum* L., provided by Buck Co.) were surface sterilized with NaClO at 50% (v/v) (4% active Cl_2) for 10 min and then thoroughly rinsed with sterile distilled water. They were germinated in plastic pots (1 l) filled with vermiculite and irrigated with Hoagland solution (Hoagland & Arnon 1950) for 4 weeks. Plants were grown at 26/20 °C (day and night), with a 16-h photoperiod under fluorescent white light (175 μmol/m^2/s) in a controlled environmental growth chamber. Leaf segments (8 mm length) from 30-day-old plants were cut with scissors, put in flasks containing 25 ml of 1 mM Spm or distilled water and incubated during 6 h in a rotatory shaker under continuous illumination. After this preincubation, leaf segments were transferred to distilled water, 0.5 mM $CdCl_2$ or 0.5 mM $CuCl_2$ and incubated during 14 h more in the same incubation conditions. Treatments were as follows: (a) Controls in distilled water (C); (b) distilled water–0.5 mM $CdCl_2$ (Cd); (c) distilled water–0.5 mM $CuCl_2$ (Cu); (d) 1 mM Spm–distilled water (Spm); and (e) 1 mM Spm–0.5 mM $CdCl_2$ or 0.5 mM $CuCl_2$ (Spm + Cd or Spm + Cu). After incubation, leaf segments were thoroughly washed with distilled water and extracted for analysis.

Cadmium, copper and Spm concentrations used for treatments were selected after previous experiments in our laboratory or according to concentrations found in literature (Groppa *et al*. 2001; Shen *et al*. 2000).

Analysis of Pas

Plant tissues (300 mg FW) were homogenized with 5% (v/v) perchloric acid, kept 30 min on ice and centrifuged at 5000 rpm for 10 min. The supernatants were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1,6 hexanediamine was used as an internal standard. Standards of Put, Spd and Spm were dansylated simultaneously. The dansylated derivatives were extracted with 1 ml ethylacetate. Pas were separated and identified by TLC, performed on high-resolution silica gel plates (JT Baker, silica gel plates IB 2-F) using *n*-hexane:ethyl acetate (1:1) solvent system. Dansylated Pas were identified by comparing the R_f values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the Pas in the samples and standards were scraped off the plates and eluted with 1 ml ethylacetate. Their fluorescence was measured at 365 nm excitation and 510 nm emission, in a spectrofluorometer (Aminco Bowman).

Determination of ADC, ODC and DAO activities

All assays were performed on fresh extracts according to the method described by Flores and Galston (1984). Samples (300 mg FW) were homogenized in a chilled mortar with 2 ml of 50 mM phosphate buffer (pH 7.8) containing 0.5 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF and 1 mM pyridoxal phosphate. They were centrifuged at 15,000 rpm for 20 min and the supernatants were immediately used for enzyme assays. The incubation mixture for ADC (EC 4.1.1.19) consisted of 100 μl of the crude extract, 70 μl of buffer, 10 mM pyridoxal phosphate, 25 mM dithiothreitol, and the substrate [1-^{14}C] arginine (325 mCi/mmol, New England Nuclear) diluted with cold arginine to give a final concentration of 20 mM, in a final reaction volume of 200 μl. ODC (EC 4.1.1.17) was assayed in a similar way using [1-^{14}C] ornithine (54.3 mCi/mmol, New England Nuclear), diluted with cold ornithine to give a final concentration of 20 mM. Specific activities of the enzymes were expressed as nmol $^{14}CO_2$/h/g FW. Reaction mixtures were incubated for 60 min at 37 °C under continuous shaking. The reaction was stopped adding 100 μl of trichloroacetic acid (TCA) 20% (w/v) and the incubation continued for 45 min. For blanks, TCA was added at zero time. The $^{14}CO_2$ released in the reaction was trapped in Whatmann filter papers moistened with Protosol (New England Nuclear) and placed above the reaction mixture in glass tubes similar to the plastic wells of Kontes. When the reaction was finished, the filter papers were put in a scintillation solution in glass vials and the radioactivity was measured in a Beckmann LS 1801 scintillator counter.

DAO (EC 1.4.3.6) activity was assayed using 200 μl of crude extract, 50 μl of 100 mM Tris–HCl buffer, and 40 μl of [1-^{14}C] Put (110 mCi/mmol), New England Nuclear) diluted with cold Put to final reaction volume of 290 μl. Reaction mixture was incubated for 60 min at 37 °C under continuous shaking. The ^{14}C-labeled pyrroline produced was extracted with 3 ml toluene from the reaction mixture after addition of 200 μl of $NaCO_3$ to bring the pH to 8.0. For blanks, TCA was added at zero time. One milliliter of toluene extract was placed in a scintillation solution in glass vials and the radioactivity was measured in a Beckmann LS 1801 scintillator counter (Martin-Tanguy *et al.* 1997).

Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was determined as the amount of TBARS, measured by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated leaf fragments (300 mg FW) were homogenized in 3 ml of 20% (w/v) TCA. The homogenate was centrifuged at 15,000 rpm for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA and 100 μl 4% (w/v) BHT in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 3000 rpm for 3 min and the absorbance was measured at 532 nm. The concentration of TBARS was calculated using an extinction coefficient of 155 mM/cm.

In situ H_2O_2 localization

In situ H_2O_2 production was detected by an endogenous peroxidase-depending staining procedure using 3,3′ diaminobenzidine (DAB) (Thordal-Christensen *et al.* 1997). Leaves segments were

immersed in a 1 mg/ml DAB solution, pH 3.8, vacuum-infiltrated for 5 min, incubated at room temperature for 1 h under dark, and then illuminated until appearance of brown spots to evidence H_2O_2 formation. Leaves were bleached in boiling ethanol.

Enzyme preparations and assays

Extracts for determination of APOX, unspecific peroxidases (GPOX) and SOD activity were prepared from 300 mg FW of leaves homogenized under ice-cold conditions in 3 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g polyvinylpyrrolidone, and 0.5% (v/v) Triton X-100 at 4 °C. Because APOX is labile in the absence of ascorbate, 5 mM ascorbate was included for the extraction of this enzyme. The homogenates were centrifuged at 15,000 rpm for 20 min and the supernatant fraction was used for the assays. APOX activity (EC 1.11.1.11) was measured immediately in fresh extracts as described by Nakano and Asada (1981), using a reaction mixture (1 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε: 2.8 mM/cm). Total SOD (EC 1.15.1.1) activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana *et al.* (1986). The reaction mixture contained 50–150 μl of the plant extract and 3.5 ml O_2^- generating solution which contained 14.3 mM methionine, 82.5 μM NBT, and 2.2 μM riboflavin. Extracts were brought to a final volume of 0.3 ml with 50 mM K-phosphate (pH 7.8) and 0.1 mM EDTA. Test tubes were shaken and placed at 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was followed by obtaining the A_{560} values. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as amount of enzyme which produced a 50% inhibition of NBT reduction under the assay condition. GPOX activity (EC 1.11.1.7) was determined in a reaction mixture containing 50 mM phosphate buffer (pH 7.4), 1 mM guaiacol, 0.1 mM H_2O_2 and 100 μl enzyme preparation in a final volume of 1 ml. Activity was measured by following the increase in absorbance at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM/cm) (Maehly & Chance 1954). Extracts for determination of GR (EC 1.6.4.2) activity were prepared from 300 mg FW of leaves homogenized under ice-cold conditions in 3 ml of extraction buffer containing 50 mM Tris–HCl buffer (pH 7.6), and 1 mM EDTA. GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation (Schaedle & Bassham 1977). The reaction mixture contained leaf extract, 1 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 50 mM Tris–HCl buffer (pH 7.5) and 3 mM $MgCl_2$.

Glutathione determination

Non-protein thiols were extracted by homogenizing 300 mg FW of leaf fragments in 2 ml of 5% (w/v) sulfosalycilic acid (pH 2). After centrifugation at 15,000 rpm for 20 min at 4 °C, the supernatants were used for the analysis. Total glutathione (GSH plus GSSG) was determined measuring the absorbance increment at 412 nm, using GR, DTNB and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG (Anderson 1985).

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Cadmium and copper effect on Pas level

The content of the three measured Pas, Put, Spd and Spm was significantly altered after Cd^{2+} and Cu^{2+} treatments. Cadmium and copper increased Put content by 282% and 89%, respectively. Spd content remained unaltered either with Cd^{2+} or Cu^{2+}, while Spm was reduced to 62% and 53% of control values by cadmium and copper treatments,

Table 1. Effect of 0.5 mM $CdCl_2$ or $CuCl_2$ on Pas content.

Treatment	Putrescine (nmol/g FW)	Spermidine (nmol/g FW)	Spermine (nmol/g FW)
C	44.94 ± 5.47[a]	46.75 ± 10.36[a]	46.87 ± 4.39[a]
Cd	171.94 ± 24.93[c]	44.04 ± 6.89[a]	28.86 ± 3.99[b]
Cu	85.06 ± 6.86[b]	38.21 ± 7.71[a]	24.79 ± 2.39[b]
Spm	259.29 ± 96.84[c]	162.01 ± 30.48[b]	2791.76 ± 198.59[c]
Spm + Cd	250.78 ± 26.01[c]	136.58 ± 35.45[b]	2196.66 ± 190.64[c]
Spm + Cu	168.27 ± 34.21[c]	160.37 ± 12.23[b]	2081.21 ± 32.47[c]

Leaf segments were incubated for 14 h under continuous light with 0.5 mM $CdCl_2$ or $CuCl_2$ after 6 h preincubation with distilled water or 1 mM Spm, as described in Materials and Methods. Values are the means of two different experiments with four replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

respectively (Table 1). Spm pretreatment applied to leaf segments resulted in elevated levels of this polyamine in the leaf segments, confirming that it was effectively transported and accumulated in the tissues.

Cadmium and copper effect on Put biosynthetic enzymes

The activity of ADC and ODC, the two biosynthetic enzymes that lead to Put formation in plants, were measured in cadmium- and copper-treated wheat leaf segments. As shown in Table 2, ADC and ODC activities were increased by cadmium 3-fold and 23-fold, respectively. Copper increased only ODC activity by about 5.5-fold over the controls. Although exogenous addition of Spm alone did not modify the activity of the biosynthetic enzymes compared to controls, the polyamine inhibited the metals-induced increase in ADC and ODC activities, returning ADC activity to values even lower than controls, and ODC activity to values slightly higher than controls, when it was added to the incubation medium previously to the metals treatments (Table 2).

Metal effect on DAO activity

Both metals diminished DAO activity in wheat leaf segments. Cadmium produced a reduction of 32% of DAO activity, while copper reduced the enzyme activity by 60% respect to the control values (Table 2). Preincubation with Spm 1 mM did not modify the effect produced by the metals on the enzyme activity.

In order to measure PAO activity, we tested several protocols, but the activity of the enzyme could not be detected in neither of the treatments applied on leaf segments (data not shown). The impossibility to detect PAO could be due to a very low activity of the enzyme in this experimental system or because the sensitivity of the methods used were not enough to measure a very low enzyme activity.

TBARS

TBARS were measured as markers of lipid peroxidation in leaf segments. Cadmium increased TBARS content 36% and copper 104% respect to the controls (Figure 1). Spm protected cell mem-

Table 2. Effect of 0.5 mM $CdCl_2$ or $CuCl_2$ on ADC and ODC activities.

Treatment	ADC (nmol $^{14}CO_2$/h/g FW)	ODC (nmol $^{14}CO_2$/h/g FW)	DAO (nmol ^{14}pyrroline/h/g FW)
Control	37.54 ± 3.11[a]	6.94 ± 0.33[a]	75.53 ± 12.4[a]
Cd	115.07 ± 8.57[c]	160.70 ± 12.54[d]	50.25 ± 8.5[b]
Cu	33.24 ± 4.58[a]	39.47 ± 7.14[c]	33.11 ± 4.4[c]
Spm	30.73 ± 4.22[a]	6.29 ± 0.08[a]	80.44 ± 10.2[a]
Spm + Cd	21.25 ± 3.19[b]	8.56 ± 0.41[b]	45.26 ± 3.2[b]
Spm + Cu	20.03 ± 1.38[b]	8.36 ± 0.18[b]	39.42 ± 3.3[c]

Leaf segments were incubated for 14 h with 0.5 mM $CdCl_2$ or $CuCl_2$ after the incubation with distilled water or 1 mM Spm. Enzymatic activities were assayed as described in Materials and methods. Data are the means ± S.E. of two different experiments with five replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

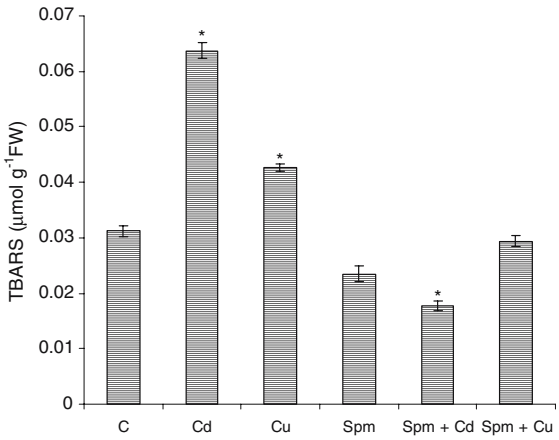

Figure 1. TBARS changes in wheat leaf segments treated with 0.5 mM $CdCl_2$ or $CuCl_2$. Leaf segments were incubated for 14 h under continuous light. Spm pretreatment consisted of 6 h preincubation with 1 mM Spm before metal incubation. Values are the means of two different experiments with three replicated measurements, and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test.

branes against metals-induced oxidative damage and this protection was evidenced by the reduction on TBARS content when leaf segments were incubated with the polyamine previously to the metal treatments (Figure 1). Spm by itself diminished TBARS content by 25%. This polyamine completely reverted cadmium- and copper-induced lipid peroxidation, returning TBARS content to the control levels.

H_2O_2 formation

To determine if 0.5 mM Cd^{2+} or Cu^{2+} induced the "*in situ*" H_2O_2 accumulation in leaf segments, a histochemical method was used, using DAB to visualize the deposition of H_2O_2 as brown spots on the tissues due to the formation of formazan. The results are shown in Figure 2. Leaf segments treated with Cu^{2+} showed a more intense deposition of formazan, suggesting a substantial higher H_2O_2 content in these tissues compared to Cd^{2+}-treated ones. The H_2O_2 formation was partially reduced by Spm pretreatment, as observed in the figure, and this effect was more evident in Cu^{2+}-treated tissues, as visualized by the intensity of the brown spots. The polyamine by itself did not produce any effect on H_2O_2 formation.

APOX, GPOX, GR and SOD activities

APOX, GPOX, SOD and GR were the enzymes selected to evaluate the oxidative damage caused by cadmium and copper on the antioxidant defense system. APOX activity was reduced to 64% and 31% of the controls, by Cd^{2+} and Cu^{2+}, respectively (Table 3). Unexpectedly, Spm by itself also decreased the activity of this enzyme by 31%, thus being ineffective in protecting APOX against

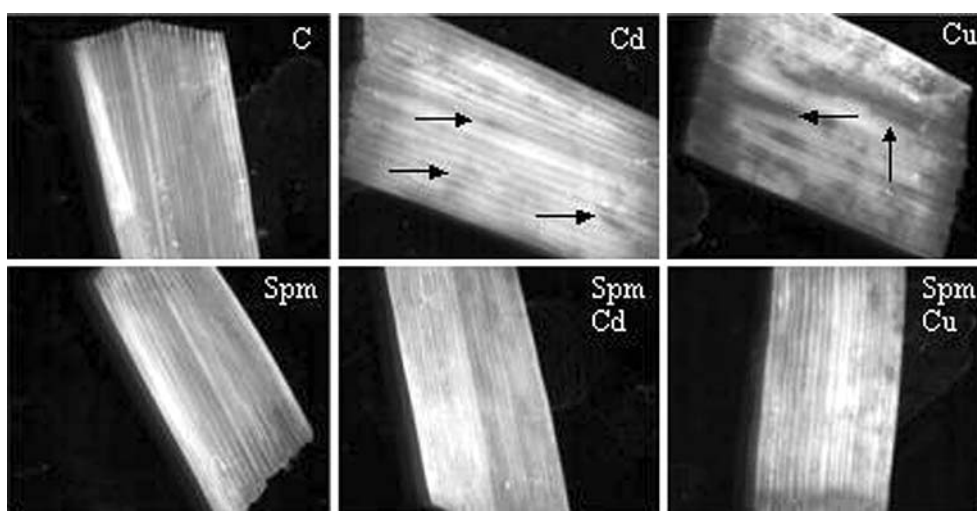

Figure 2. Histochemical detection of H_2O_2 in wheat leaf segments. Leaf segments were incubated for 14 h under continuous light. Spm pretreatment consisted of 6 h preincubation with 1 mM Spm before metal (Cd or Cu) incubation. Treatments are detailed in the figure. Leaves were infiltrated with 1 mg/ml DAB for 1 h under dark, and then illuminated until appearance of brown spots (see arrows) to evidence H_2O_2 formation. To visualize DAB deposits, leaves were bleached in boiling ethanol. The figure is representative of three different experiments.

Table 3. Effect of 0.5 mM $CdCl_2$ or $CuCl_2$ on APOX, GPOX, GR and SOD enzyme activities.

Treatment	APOX[A] (U/g^{-1} FW)	GPOX[B] (U/g^{-1} FW)	GR[C] (U/g^{-1} FW)	SOD[D] (U/g^{-1} FW)
C	5.72 ± 0.42[a]	4.60 ± 0.23[a]	0.300 ± 0.038[a]	30.89 ± 1.23[a]
Cd	3.68 ± 0.30[b]	4.11 ± 0.23[a]	0.153 ± 0.029[b]	31.15 ± 1.93[a]
Cu	1.77 ± 0.28[c]	4.21 ± 0.42[a]	0.127 ± 0.022[b]	228.81 ± 98.25[b]
Spm	3.94 ± 0.33[b]	3.69 ± 0.21[a]	0.301 ± 0.022[a]	20.84 ± 4.43[c]
Spm + Cd	4.09 ± 0.56[b]	4.13 ± 0.31[a]	0.353 ± 0.027[a]	16.39 ± 1.12[c]
Spm + Cu	1.05 ± 0.20[c]	3.56 ± 0.68[a]	0.206 ± 0.030[c]	30.07 ± 2.57[a]

Wheat leaf segments were incubated for 14 h with 0.5 mM $CdCl_2$ or $CuCl_2$ after the incubation with distilled water or 1 mM Spm. Enzymatic activities were assayed as described in Materials and Methods. Data are the means ± S.E. of two different experiments with five replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.
[A]One unit of APOX forms 1 μmol of ascorbate oxidized min^{-1} under the assay conditions.
[B]One unit of GPOX produces 1 μmol of tetraguaiacol min^{-1} under the assay conditions.
[C]One unit of GR oxidizes 1 μmol of NADPH min^{-1} under the assay conditions.
[D]One unit of SOD is the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions.

the metals-induced oxidative damage, reducing the enzyme activity even more when was added previously to copper treatment (Table 3). GPOX activity was not affected by neither of the treatments used nor by Spm alone. GR activity, that catalyzes the NADPH-dependent reduction of oxidized glutathione, was reduced by both Cd^{2+} and Cu^{2+} to 50% and 58% of the controls, respectively. However, though Spm completely restored GR activity to the level of controls in Cd^{2+}-treated plants, the restoration of the enzyme activity in Cu^{2+}-treated plants reached only 68% of the control (Table 3). Spm alone did not modify the enzyme activity respect to controls. Opposite to the results observed with APOX and GR, copper increased SOD activity 7-fold respect to the controls while cadmium did not modify the enzyme activity (Table 3). The addition of Spm previously to the metal treatments reverted the increase produced by Cu^{2+}, returning the enzyme activity to control levels (Table 3).

Glutathione content

Glutathione was measured in order to analyze the effect of the metals tested on a soluble antioxidant compound. In wheat leaf segments, glutathione was reduced 62% by cadmium and 77% by copper, as compared to controls (Figure 3). Spm previously added to the medium did not restore the diminished GSH level produced by both metals. This polyamine did not affect GSH levels when was added alone to the incubation medium (Figure 3). The concentration of oxidized glutathione (GSSG) showed almost no variation either with Cd^{2+} or Cu^{2+} and remained between 8% and 12% of total glutathione in all treatments (data not shown), so only GSH variation was shown.

Discussion

Many authors have reported increased levels of Pas when plants are exposed to diverse kind of environmental stress (Bouchereau *et al.* 1999; Walters 2003; Nayyar & Chander 2004). In accordance with the results found by Weinstein

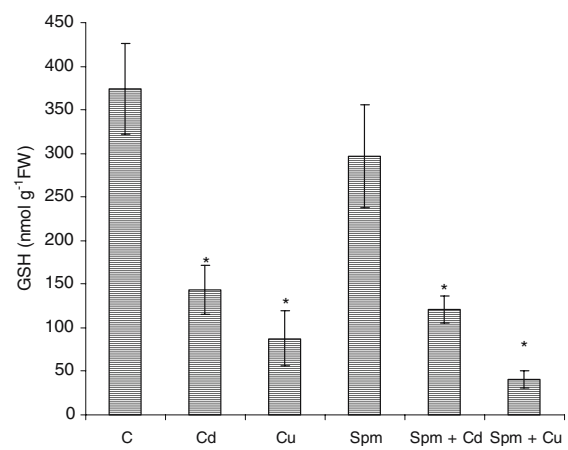

Figure 3. Cadmium and copper effect on glutathione (GSH) content. Leaf segments were incubated for 14 h under continuous light with 0.5 mM $CdCl_2$ or $CuCl_2$ after 6 h incubation with distilled water or 1 mM Spm, as described in Materials and methods. Values are the means of two different experiments with three replicated measurements, and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test.

et al. (1986) in oat and bean plants treated with cadmium, we found that cadmium and copper significantly increased Put content in wheat leaf segments. However, in a previous work of our group (Groppa *et al*. 2001), we reported a decrease in Put level in Cd^{2+}- or Cu^{2+}-treated sunflower leaf disks, using the same experimental design. This data clearly support the idea that polyamine metabolism is not only related to the environmental conditions but also to the plant species. Besides, it has been reported that, mainly in cereals, Put accumulation seemed to be related specially to an activation of ADC under stress conditions (Flores & Galston 1984; Bouchereau *et al*. 1999; Martin-Tanguy 2001). Our results showed that Put accumulation in cadmium-treated wheat leaves was mediated by a simultaneous enhancement of ADC and ODC activities, but only ODC was responsible for the increased Put levels in copper-treated leaf disks. Das *et al*. (1995) reported that both ADC and ODC were responsible for Put accumulation in *Brassica campestris* seedlings under salt stress. In wheat leaves, the increase in Put content under both metals treatments could also be due to an inhibition of DAO activity, which lead to a lower degradation of this polyamine.

Spd level was not modified by any of the metals, while Spm decreased to a similar extent under Cd^{2+} or Cu^{2+} stress. However, previous results of our group using sunflower leaf disks showed a significant decreased in Spd content and no variation in Spm level when they were treated by Cd^{2+} or Cu^{2+} (Groppa *et al*. 2001), respectively. The reasons for Spm decline, despite Put accumulation in Cd^{2+}- or Cu^{2+}-treated wheat leaves, might be attributed to a higher degradation of the polyamine by PAO or to an increased ethylene formation due to an increased SAM flux into ethylene biosynthesis, in detriment of that of higher Pas. Consequently, there was an inhibition in the conversion of Put to Spd or Spm, despite Put availability. Unfortunately, we were unable to detect PAO activity in our experimental system, though we tested several protocols. Jacobsen *et al*. (1992) reported no changes in Spd or Spm content in chromium-exposed leaves of barley and rape plants, but Put accumulated with increasing chromium concentrations or exposure time. Lin and Kao (1999) reported increased Put, no significant changes in Spd and a decrease in Spm concentration in rice leaves, but using very high concentrations of copper in the assay (1 and 10 mM).

Spm has been reported to function as a free radical scavenger (Ha *et al*. 1998) and to be capable of quenching chemically generated singlet oxygen, protecting DNA from reactive oxygen species damage (Drolet *et al*. 1986; Khan *et al*. 1992). Considering that the plant plasma membrane may be regarded as the first structure that is target for metal toxicity and since Spm demonstrated a certain antioxidant capability by reducing TBARS formation in diverse systems (Benavides *et al*. 2000; Groppa *et al*. 2001), we tested this polyamine to evaluate its antioxidant properties in wheat leaves. In this experimental system, Spm completely reverted Cd^{2+}- or Cu^{2+}-induced increase of lipid peroxidation, suggesting that its protective role against the oxidative damage produced by these metals could be related to an avoidance of the membrane damage produced by an increase free radicals formation. On the other hand, the effect of the amine in the reversal of ADC, ODC and SOD activities to the level of controls seemed to be more related to the lower stress experienced by the tissues due to a lower entrance of the metals when Spm was supplied previously to the metals treatment. Alternatively, Nagele *et al*. (1994) reported that endogenous or constitutive Put can affect the redox status of the plant cells. The higher level of Put observed in Cd^{2+}-treated leaves might be contributing to a higher protection exerted against the oxidative damage, as evidenced by a lower level of TBARS and a less decay in APOX activity observed upon Cd^{2+} treatment. However, when Put was added exogenously, it did not show any remarkable antioxidant effect (data not shown). This result might be indicating that it might not be appropriate to compare the effect exerted by endogenous or constitutive Pas levels to the effect produced by exogenously added Pas, mainly because supplied Pas allocated preferentially in the apoplastic space and do not freely cross membranes, while endogenous amines were widely distributed in the cell.

It has also been postulated that Pas could act as metal chelators (Lovaas 1997; Lomozik *et al*. 2005), thus reducing the metals entrance to the cell. Spm was able to reduce the metals entrance to the tissues (data not shown), but this reduction was not enough to avoid GSH depletion. Glutathione seems to play a very important role in

plants exposed to Cd^{2+}, considering that it is the monomer of the phytochelatin molecule that can form complexes with cadmium and sequester it into the vacuoles (Bruns *et al.* 1997), and through a direct binding of Cd^{2+} to GSH (Sanità di Toppi & Gabrielli 1999). The metal-induced depletion of GSH have been reported in different plant species and experimental designs (Gallego *et al.* 1996; Benavides *et al.* 2000; Dixit *et al.* 2001). In wheat leaf segments GSH was significantly reduced by both metals, possibly due to a high and continuous utilization of GSH for phytochelatin synthesis and Spm antioxidant behavior was not enough to avoid this depletion. However, Spm was effective in restoring GSH content in paraquat-treated sunflower leaves (Benavides *et al.* 2000), suggesting that the antioxidant efficiency could be related to the type of free radicals involved, which could be different under both kind of stress. Both metals reduced the activity of APOX and GR. Spm by itself reduced APOX activity, thus evidencing no effect as antioxidant in relation to this enzyme, as was reported for sunflower leaf disks (Groppa *et al.* 2001). Pas have been shown to bind to many kind of proteins (Carley *et al.* 1983) and probably a direct inhibition of APOX activity by Spm was taking place. However, Cd^{2+}- and Cu^{2+}-diminished GR activity was completely or partially restored by this polyamine, respectively. The maintenance of high GR activity is essential to maintain a high level of GSH. However, though Spm reverted enzyme activity to the control level, it could not modify the metal-induced decrease in GSH content.

GPOX activity was not affected by the metals while SOD activity increased only with copper treatment and this increase was reversed by Spm pretreatment. It should be interesting to check if the increased SOD activity observed under Cu stress could be related to an specific increase in the activity of any of the isoforms of the protein, i.e. the Cu–Zn isoform located in chloroplasts and cytosol. However, the increased total SOD activity observed in radish in response to Cd treatment was not correlated with any specific SOD isoform (Vitória *et al.* 2001).

Several mechanisms have been suggested to explain the increased oxidant resistance attributed to Pas, i.e., Pas could act as direct radical scavengers (Drolet *et al.* 1986); they could bind to antioxidant enzymes or be conjugated to antioxidant molecules and allow them to permeate to the sites of oxidative stress (Poduslo & Curran 1996); or may interact with membranes stabilizing molecular complexes of thylakoid membranes (Besford *et al.* 1993). Plasma membrane function may be rapidly affected by heavy metals (Quartacci *et al.* 2001). The antioxidant protection exerted by exogenous Spm on wheat leaves seemed to be related to a protection of the membrane integrity through lipid stabilization and avoidance of leakage of solutes in Cd^{2+}- or Cu^{2+}-induced oxidative damage. On the other hand, Kurepa *et al.* (1998) reported that paraquat resistance did not necessarily correlate with increased Pas content.

Lovaas (1997) suggested that the antioxidative effect of Pas is due to a combination of their anion and cation-binding properties. The binding of Pas to anions (phospholipid membranes, nucleic acids) contributes to a high local concentration at particular sites prone to oxidations, whereas the binding to cations efficiently prevents the site-specific generation of ''active oxygen'' (hydroxyl radicals and singlet oxygen).

The main findings of our results point out that polyamine metabolism is largely dependent on the plant specie when Cd^{2+} and Cu^{2+} were used as stressors, as suggested by the results obtained in wheat and sunflower plants (Groppa *et al.* 2001) and suggest that Spm was implicated in the protection against the metals-induced oxidative damage in wheat leaves, through the reduction of the metals-induced TBARS and H_2O_2 formation and the recovery of GR activity when applied exogenously before Cd^{2+} or Cu^{2+} treatments. However, whereas its antioxidant role is unambiguous in relation to the protection of these parameters, it was not effective in the restoration of APOX activity or the maintenance of a high SOD activity, the latter in the case of Cu^{2+} stress. The elucidation of the relationship between Pas and certain antioxidant enzymes will shed light with respect to the precise mechanism by which Spm exert its antioxidant role.

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