BIOLOGIA PLANTARUM 42 (1): 49-55, 1999

Effect of cadmium ions on antioxidant defense system in sunflower cotyledons

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Abstract

Sunflower (*Helianthus annuus* L.) seeds were germinated and grown in the presence of 50, 100 and 200 μM $CdCl_2$. The lower concentration (50 μM) of Cd^{2+} ions produced slight decrease in reduced glutathione (GSH) content and overall increase (except superoxide dismutase) in antioxidant enzyme activities, and in H_2O_2 concentration. Chlorophyll content, lipid peroxidation and protein oxidation were not affected under 50 μM $CdCl_2$. GSH content was diminished under 100 and 200 μM $CdCl_2$, and except for superoxide dismutase, which activity remained unaltered, overall decreases in the antioxidant enzyme activities (catalase, ascorbate peroxidase, dehydroascorbate peroxidase, glutathione reductase) and in guaiacol peroxidase were observed. These Cd^{2+} concentrations caused a decrease in chlorophyll content as well as an increase in lipid peroxidation, protein oxidation and H_2O_2 concentration. All the observed effects were more evident with the highest concentration of cadmium chloride used.

Additional key words: chlorophyll, glutathione, *Helianthus annuus*, lipid peroxidation, oxidative stress.

Introduction

Environmental biotic and abiotic factors, including excess of metal ions, induce oxidative stress by increasing the formation of active oxygen species such as $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ in plant cells (Van Assche and Clijsters 1990, Scandalios 1993, Luna

Received 16 June 1998, *accepted* 20 September 1998.

Abbreviations: APOX - ascorbate peroxidase; CAT - catalase; DHAR - dehydroascorbate reductase; GPOX - guaiacol peroxidase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; SOD - superoxide dismutase.

Acknowledgements: This work was supported by grants from the Universidad de Buenos Aires (Argentina) and from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Argentina). M.L.T. and M.P.B. are career investigators from CONICET and S.M.G. is fellow from CONICET.

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et al. 1994, Stohs and Bagchi 1995, Gallego *et al.* 1996). Plants have developed a complex antioxidant system which includes GSH, ascorbic acid, α-tocopherol, carotenoids and enzymes that protect the plant against oxidative damage. Superoxide dismutases (SOD) are a group of enzymes which accelerate the conversion of O_2^- to H_2O_2 (Salin 1987). Catalase (CAT), ascorbate peroxidase (APOX), and a variety of general peroxidases catalyze the breakdown of H_2O_2. In the ascorbate-glutathione cycle, the enzymatic action of APOX produces monodehydroascorbate radical which can dismutate spontaneously to ascorbate and dehydroascorbate or be enzymatically reduced to ascorbate by NADPH-dependent monodehydroascorbate reductase (MDHAR). Also, dehydroascorbate is reduced to ascorbate enzymatically in a reaction mediated by GSH-dependent dehydroascorbate reductase (DHAR). The resulting GSSG is then converted back to the reduced form (GSH) by a NADPH dependent glutathione reductase (GR). GR also plays an essential role in the protection of chloroplasts against oxidative damage by maintaining a high GSH/GSSG ratio (Esterbauer and Grill 1978, Pastori and Trippi 1992). The oxidative damage caused by several metals, such as Cu and Fe, can be explained directly as involving changes in redox state (Fenton reaction). The molecular mechanisms of Cd^{2+} toxicity are not known. So far, the influence of Cd^{2+} on the active oxygen species in plant tissues has been described in only a few reports (Hendry *et al.* 1992, Somashekaraiah *et al.* 1992, Gallego *et al.* 1996, Stroinski and Zielezinska 1997, Stroinski and Kozlowska 1997). Sunflower is one of the major crops world-wide for oil and protein production and it has been demonstrated that *Helianthus annuus* L. could be used to reduce metal concentrations in the environment (Salt *et al* 1995).

In order to shed light on the toxicity resulting from Cd exposure, we have carried out a study about the variation of the antioxidant defense system and the oxidative stress generation in cotyledons of sunflower growing in the presence of different cadmium concentrations.

Materials and methods

Plants and growing condition: Seeds of sunflower (*Helianthus annuus* L., cv. Mycosol 2, supplied by *Agrigenetics*, Buenos Aires, Argentina) were germinated and grown on filter paper in plastic dishes containing aqueous solution of 0 (control), 50, 100 and 200 μM $CdCl_2$. Samples for analysis were taken from cotyledons of plants that were grown 96 h in a controlled climate room at temperature of 24 + 2 °C, relative humidity of 50 %, 16-h photoperiod and an irradiance of 175 μmol(photon) m^{-2} s^{-1}. Each experiment was repeated three times and consisted of five replicates.

Analytical assays: Chlorophyll content was determined by the method described by Wintermans and De Mots (1965). Lipid peroxidation was quantified as the concentration of thiobarbituric acid reactive substances (Minotti and Aust 1987). Protein oxidation was measured as the total content of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (Levine *et al.* 1990). Total glutathione (GSH plus GSSG) was determined in homogenates spectrophotometrically at 412 nm, using

glutathione reductase, 5,5' dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated as the difference between total glutathione and GSSG (Anderson 1985). Hydrogen peroxide content was measured as described by Guilbault *et al.* (1968). Protein concentration was evaluated by the method of Bradford (1956) using bovine serum albumin as the standard.

Enzyme assay: Extracts for enzyme determinations were made as described elsewhere (Gallego *et al.* 1996). CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm (Chance *et al.* 1979). Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described by Becana *et al.* (1986). One unit of SOD was defined as amount of enzyme which produced a 50 % inhibition of NBT reduction under the assay condition. APOX activity was measured immediately in fresh extracts according to Nakano and Asada (1981). The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm. GPOX activity was determined as described by Nakano and Asada (1981). Activity was measured by following the increase in absorbance at 470 nm. GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation (Shaedle and Bassham 1977). DHAR activity was measured by formation of ascorbate at 265 nm, as described by Nakano and Asada (1981).

Statistical analysis: Figures in the text and tables indicate mean values ± SE. Differences between control and treated plants were analysed by one-way *ANOVA*, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results and discussion

Compared to controls, GSH content was slightly decreased (10 %) with 50 μM Cd^{2+}, however, 100 and 200 μM Cd^{2+} caused a marked decrease in GSH content (25 and 40 %, respectively) (Table 1). GSSG content was also diminished, (10, 25 and 40 % decrease at 50, 100 and 200 μM, respectively). After Cd^{2+} treatment no

Table 1. Effect of different cadmium chloride concentrations on glutathione content. Plants were treated for 96 h with 50, 100, and 200 μM $CdCl_2$. Means ± SE of three different experiments with five replicated measurements. Different letters mean significant differences at $P < 0.05$ according to Tukey's multiple range test.

$CdCl_2$ [μM]	GSH [nmol g^{-1} (f.m.)]	GSSG [nmol g^{-1} (f.m.)]	GSH/GSSG
0	$16.1 + 0.2^a$	$2.0 + 0.1^a$	8.0 ± 0.2^a
50	14.4 ± 0.2^b	1.8 ± 0.1^b	8.0 ± 0.2^a
100	12.1 ± 0.2^c	1.5 ± 0.1^c	8.1 ± 0.2^a
200	$9.6 + 0.2^d$	$1.2 + 0.1^d$	8.0 ± 0.2^a

changes in the GSH/GSSG ratio was observed (Table 1). A marked GSH decrease could be due to a strong increase in H_2O_2 concentration (Fig. 1*D*) in agreement with the results obtained by Stroinski and Zielezinska (1997). No synthesis of phytochelatins was observed after 96 h Cd^{2+} exposure (data not shown).

Despite SOD is considered as a key antioxidant, cadmium treatments did not produce changes in SOD activity in our experimental system (Table 2). In agreement, SOD seems to play a minor role in sunflower seed ageing (Bailly *et al.* 1996) and SOD activity is not always the most important factor for the tolerance of the plant cells to oxidative stress (Stroinski and Kozlowska 1997). Significant increments for CAT, APOX, DHAR, and GR (20, 18, 22, and 20 %, respectively, with respect to control values) were obtained under 50 μM Cd^{2+} treatments (Table 2). In contrast, antioxidant enzyme activities were decreased by two higher concentrations of $CdCl_2$ (Table 2). CAT was drastically diminished with respect to control values with both, 100 and 200 μM Cd^{2+} treatments (48 and 57 %, respectively), APOX decreased 24 and 32 % for 100 and 200 μM of $CdCl_2$, respectively (Table 2). Treatments with 100 μM Cd^{2+} produced a moderate decrease in DHAR (33 %) and GR (25 %) activities while 200 μM Cd^{2+} caused a 45 % decrease for DHAR and 49 % decrease for GR, with respect to control values (Table 2). Similar to CAT and APOX, GR could also play a part in the control of endogenous H_2O_2 content through an oxido-reduction cycle involving glutathione and ascorbate, and its role in H_2O_2 detoxification has been demonstrated in plants (Dalton *et al.* 1987).

Table 2. Effect of 50, 100, and 200 μM $CdCl_2$ on activity of SOD [U mg^{-1}(protein)], CAT [pmol mg^{-1}(protein)], APOX [U mg^{-1}(protein)], DHAR [U mg^{-1}(protein)], GR [U mg^{-1}(protein)] and GPOX [U mg^{-1}(protein)]. Plants were treated for 96 h. Means ± SE of three different experiments with five replicated measurements. Different letters mean significant differences at $P < 0.05$. One unit of APOX forms 1 nmol of ascorbate oxidized s^{-1}, one unit of DHAR forms 1 nmol of ascorbate s^{-1}, one unit of GR oxidizes 1 nmol of NADPH s^{-1}, one unit of GPOX oxidizes 1 nmol of guaiacol s^{-1}.

$CdCl_2$	SOD	CAT	APOX	DHAR	GR	GPOX
0	0.11 ± 0.01^a	0.100 ± 0.009^a	8.5 ± 0.6^a	15.2 ± 0.4^a	54.5 ± 0.5^a	30.0 ± 0.4^a
50	0.11 ± 0.01^a	0.120 ± 0.010^b	10.0 ± 0.6^b	18.5 ± 0.5^b	65.3 ± 0.6^b	35.2 ± 0.4^b
100	0.10 ± 0.01^a	0.052 ± 0.005^c	6.5 ± 0.3^c	10.0 ± 0.5^c	41.2 ± 0.5^c	12.5 ± 0.5^c
200	0.10 ± 0.01^a	0.043 ± 0.003^d	5.8 ± 0.2^d	8.5 ± 0.4^d	27.8 ± 0.4^d	10.0 ± 0.4^d

Various forms of peroxidases are also responsible for the removal of H_2O_2 from biological systems (Zhang and Kirkham 1996). To assess the behaviour of non specific peroxidases under cadmium treatment, guaiacol peroxidase activity was determined. While the 50 μM Cd^{2+} produced an 20 % increase (with respect to controls) in the enzyme activity, both 100 and 200 μM Cd^{2+}, caused a decrease in peroxidase activity (58 and 67 %, respectively) (Table 2).

Sunflower cotyledons treated with 50 μM $CdCl_2$ were not affected in their chlorophyll content, however, 100 and 200 μM $CdCl_2$ significantly diminished it (Fig. 1*A*). This effect can be explained either as a Cd^{2+}-induced direct inhibition of

chlorophyll synthesis (Van Assche and Clijsters 1990) or by oxidative stress produced by this ion (Gallego *et al.* 1996).

Reactive oxygen species are considered to be the initiators of cell damage because lipid peroxidation *in vivo* provides a steady supply of free radicals. At both higher Cd^{2+} concentrations, this parameter was significantly elevated over control values (Fig. 1*B*).

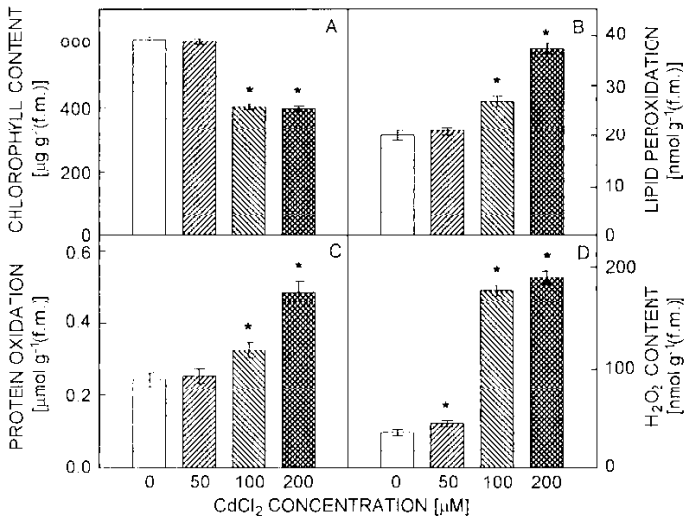

Fig. 1. Effect of different cadmium chloride concentrations on chlorophyll content (*A*), lipid peroxidation (*B*), protein oxidation (*C*) and hydrogen peroxide concentration (*D*). Lipid peroxidation was measured as thiobarbituric acid reactive substances [nmol g^{-1}(f.m.)] and protein oxidation as total carbonyl groups [µmol g^{-1}(f.m.)]. Values are the means of three different experiments with five replicated measurements, and bars indicate SE (*- significant differences at $P < 0.05$).

Protein degradation, which follows oxidative modification of proteins, has been proposed as a better index of oxidative stress than lipid peroxidation. The content of oxidatively modified proteins increased by 34 and 98 % after 96 h of 100 and 200 µM Cd^{2+} treatments compared to controls (Fig. 1*C*). Surprisingly, in the present work, no changes in total protein content were found (data not shown). Similar to chlorophyll content, treatments with 50 µM $CdCl_2$ did not produce modification either in lipid peroxidation or in protein oxidation (Fig. 1*B,C*). We have found a very high correlation between lipid and protein oxidation, indicating that both processes are intimately associated in stressed plants. H_2O_2 concentration was moderately increased (25 %) in 50 µM Cd^{2+}-treated plants (Fig. 1*D*). However, its concentration was markedly raised over control with the two higher metal ion concentrations (Fig. 1*D*). This increase could be explained by the decreased CAT, APOX and GPOX activities (Table 2), which are the enzymes responsible for the removal of

H_2O_2, in addition to an unchanged SOD activity. The increased level of H_2O_2 concentration together with the capacity of Cd^{2+} ion to induce lipid peroxidation, contributes to the increased rate of production of active oxygen species and further reduces GSH levels and antioxidant enzymes.

Summing up, lower concentrations of cadmium ions induce an antioxidant enzymatic response in sunflower cotyledons, which in turn, protect them against oxidative damage. However, the two higher cadmium concentrations produce oxidative stress.

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