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BRIEF COMMUNICATION

Antioxidative response to cadmium in roots and leaves of tomato plants

W.B. AMMAR[1]*, I. NOUAIRI[2], M. ZARROUK[2], M.H. GHORBEL[1] and F. JEMAL[1]

Unité de Recherche Nutrition et Métabolisme Azotés et Protéines de Stress, Faculté des Sciences de Tunis, Campus Universitaire, 2092 Tunis El Manar, Tunisie[1]
Laboratoire Caractérisation et Qualité de l'Huile d'Olive, Centre de Biotechnologie de Borj-Cedria B, P.901, 2050 Hammam-Lif, Tunisia[2]

Abstract

Treatment of tomato seedlings (*Lycopersicon esculentum* Mill. cv. 63/5 F1) with increasing $CdCl_2$ concentrations in the culture medium resulted in Cd accumulation more important in roots than in leaves. Biomass production was severely inhibited, even at low Cd concentration. Cd reduced chlorophyll content in leaves and enhanced lipid peroxidation. An increase in antioxidative enzyme (superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase) activities was more pronounced in leaves than in roots, while catalase activity increased only in roots. In addition, changes in isoenzyme composition were observed using the non-denaturing polyacrylamid gel electrophoresis.

Additional key words: lipid peroxidation, *Lycopersicon esculentum*, oxidative stress.

Cadmium is an important environmental pollutant with high toxicity to animals and plants. Studies carried out in different plant species have revealed that Cd causes growth inhibition (Scebba *et al.* 2006, Agrawal *et al.* 2006) and even plant death. It is well established that photosynthesis (Pietrini *et al.* 2003), mineral nutrition, membrane structure, *etc*., are affected by the presence of Cd (Ben Ammar et al. 2005). Besides, Cd induced peroxidation of membrane lipids by generating reactive oxygen species (ROS) (Dixit *et al.* 2001, Ben Youssef *et al.* 2005).

To mitigate the oxidative damage initiated by ROS, plants have developed a complex defense antioxidative system that includes low molecular mass scavengers like tocopherols, ascorbic acid and glutathione (Pinto *et al.* 2003) and enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (De Pinto *et al*. 2000). The superoxide radicals generated in plant cells are converted into H_2O_2 and O_2 by the action of SOD. The H_2O_2 produced, is eliminated by CAT, without use of an additional substrate (Pereira *et al.* 2002), and by several classes of peroxidases such as guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Kopyra and Gwóźdź 2003). Ascorbate and glutathione also, have an important role in the ascorbate-glutathione cycle (De Pinto *et al.* 2000). ASC acts directly to eliminate superoxide radicals, $^1O_2$, or superoxide, and as a secondary antioxidant during reductive recycling of the oxidized form of tocopherol. ASC oxidation always leads to monodehydroascorbate (MDA) which is normally converted to ASC by MDHAR. MDA, unless quickly reduced by MDHAR, disproportionates non-enzymatically into ASC and DHA. DHA is reduced to ASC by the action of DHAR, using GSH as the reducing substrate. The glutathione disulphide (GSSG) produced in the reaction is then reduced by glutathione reductase (GR), using NADPH as co-factor (Mittler 2002).

Cd treatment affects the activities of antioxidative enzymes, but contrasting results have been reported. For

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Abbreviations: APX - ascorbate peroxidase; Car - carotenoids, Chl - chlorophyll, CAT - catalase; DTT - dithiothereitol; EDTA - ethylenediaminetetraacetic acid; FM - fresh mass; GPX - guaiacol peroxidase; GR - glutathione reductase; GSH - glutathione reduced form; GSSG - glutathione oxidized form; MDA - malondialdehyde; NBT - nitroblue tetrazolium; POX - peroxidase; PVPP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; TBARS - thiobarbituric acid-reacting substances; TCA - trichloroacetic acid; TEMED - N,N,N0,N0-tetramethylethylenediamine.
* Corresponding author; fax: (+21) 671 885480, e-mail: benammar.wided@voila.fr

example, in leaves of pepper plants Cd increased the activity of GR and GPX, while CAT and SOD were slightly depressed (León *et al.* 2003). In contrast, in *Alyssum argenteum* and *A. maritimum*, the SOD activity was elevated at high Cd concentration, while GR activity was reduced (Schickler and Caspi 1999).

In the present work, the effects of Cd treatment on lipid peroxidation as well as on the activities of some antioxidant enzymes (SOD, CAT, APX, GPX, and GR) of tomato leaves and roots were studied.

Tomato (*Lycopersicon esculentum* Mill. cv. 63/5 F1) seeds were first sterilized in 10 % (v/v) H_2O_2, for 20 min, then, thoroughly washed with distilled water and germinated on moistened filter paper at 25 °C in the dark for 8 d. Individual plants were separated and hydroponically grown, for 10-d, in the following medium: 0.5 mM KH_2PO_4 , 1.25 mM Ca $(NO_3)_2$, 1 mM KNO_3, 0.5 mM $MgSO_4$, 100 µM Fe-SO_4-EDTA, 5 µM $MnSO_4$. 4 H_2O, 1 µM $ZnSO_4$. 7 H_2O, 1 µM $CuSO_4$. 5H_2O, 30 µM H_3BO_3, and 1 µM $(NH_4)_6Mo_7O_{24}$. 4 H_2O. Cadmium was added to the medium as $CdCl_2$ in four concentrations: 0, 1, 10, 25 and 50 µM. After 7 d of Cd treatment, young leaves (developed after Cd treatment) and roots were harvested and used for chemical analyses.

To determine the Cd content, the various plant tissues were mineralized using a HNO_3-$HClO_4$ (4:1 v/v) mixture. After mineralization the residues were solubilized in 7 % HNO_3 and Cd concentrations in the tissue extracts were measured by atomic absorption spectrophotometry (*Perkin Elmer-AAnalyst 300*, Norwalk, CT, USA). Chlorophylls and carotenoids were extracted in chloroform-methanol (v/v) mixture as described by Allen and Good (1971) and then homogenized in 80 % acetone. The resulting suspension was centrifuged for 5 min at 3 000 *g*. The pigment contents of the supernatant were estimated according to Arnon (1949). Lipid peroxide was determined by measuring the concentration of thiobarbituric acid-reacting substances (TBARS), as described by Alia *et al.* (1995). The leaves and the roots were homogenized in 5 % (m/v) trichloroacetic acid (TCA). After centrifugation, a sample of the supernatant was added to 20 % TCA containing 0.5 % (m/v) thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min. The absorbance was measured at 532 nm.

Enzyme extractions were carried out at 4 °C. The plant tissue was reduced to powder in liquid nitrogen and extracted in 50 mM potassium phosphate buffer (pH 7) containing 1 mM EDTA, 3 mM dithiothereitol (DTT) and 5 % (m/v) insoluble polyvinylpyrrolidone (PVPP). For the APX assay, 5 mM ascorbate was added to the extracted buffer. The homogenate was centrifuged at 14 000 *g* for 30 min, than the supernatant was stored in separate aliquots at -80 °C to be used for enzyme assays. CAT (EC 1.11.1.6) activity was assayed, according to Chaparro-Giraldo *et al.* (2000), by monitoring the decline in absorbance at 240 nm, as H_2O_2 was consumed. Enzyme activity was calculated using the coefficient of absorbance of 40 mM^{-1} cm^{-1}. SOD (EC 1.15.1.1) activity was measured at 560 nm according to Beyer and Fridovich (1987), based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). One unit of enzyme activity was defined as the quantity of SOD required for 50 % inhibition of NBT reduction. APX (EC 1.11.1.11) activity was assayed by following the decline in absorbance of the oxidized ascorbate at 290 nm, according to Chen and Asada (1989). Enzyme activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1} for ascorbate. GPX (EC 1.11.1.7) activity was measured according to Landberg and Greger (2002). Development of absorbance at 470 nm was measured. Enzyme activity was calculated using the coefficient of absorbance of 26.6 mM^{-1} cm^{-1} for guaiacol. GR (EC 1.6.4.2) activity was determined by monitoring the decline of absorbance at 340 nm as NADPH was oxidized (Rao *et al.* 1996). The concentration of protein in the extracts for a comparison of enzyme activity, and to ensure equal loading on the native gels was determined spectrophotometrically at 595 nm as described by Bradford (1976) with bovine serum albumin as a protein standard.

Isoforms of SOD, APX, GPX and GR were separated on nondenaturating polyacrylamide gels. Equal amounts of protein extracts were mixed with bromophenol blue and glycerol to a final concentration of 12 % (v/v) and loaded on 6 % C and 10 % T polyacrylamide gels. Gel electrophoresis was done at 4 °C for 3 h with a constant current of 30 mA. For APX, however, 2 mM ascorbate was added to the electrode buffer and the gel was pre-run for 30 min before the sample was loaded. SOD, APX and GR activities were determined on a native PAGE gels as described by Rao *et al*. (1996). GPX activity was visualized by incubating the gel in 50 mM potassium phosphate buffer (pH 7.8) containing 1 % guaiacol and 0.5 % H_2O_2 until orange band apparition.

To analyse reduced (GSH) and oxidised (GSSG) glutathione, aliquots of fresh roots and leaves were homogenised in ice-cold 10 % (m/v) TCA. The homogenate was centrifuged for 5 min at 13 000 *g* at 4 °C. GSH and GSSG contents were determined in the supernatants by the 5,5'-dithio-bis(2-nitrobenzoic acid)-GSSG reductase recycling method of Anderson (1985).

The data are presented in the figure and the table as the average of at least six replicates per treatment. Each experiment was conducted in duplicate.

Cadmium was accumulated in plant organs concomitantly with the metal concentrations in the nutrient solution. Higher proportion of cadmium was found in roots than in leaves. Cd accumulation was associated to clear symptoms of phytotoxicity, as the decrease in the dry mass production of leaves and roots and the reduction in the chlorophyll (Chl) and carotenoid (Car) contents (Table 1). Chl *a* reduction was considerably larger than that for Chl *b*. Car showed a similar pattern to that of the Chl *a*. Moreover, in roots like in leaves, Cd treatment led to a significant increase in lipid peroxidation products as a result of an oxidative stress (Table 1). The TBARS produced were dependent on Cd concentrations in the growth medium.

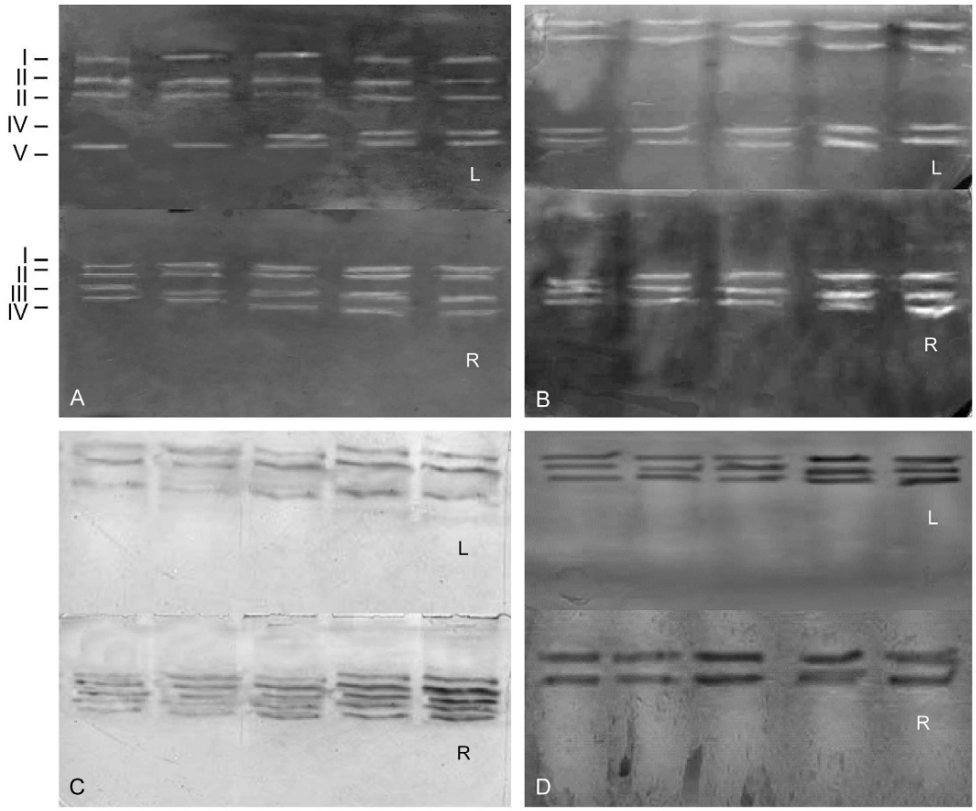

Fig. 1. Isoenzymes pattern of SOD (*A*), APX (*B*), GPX (*C*) and GR (*D*) in leaves (L) and roots (R) of tomato plants treated for 7-d with various concentrations of $CdCl_2$ (from the left to the right: 0, 1, 10, 25 and 50 μM Cd)

Table 1. Effects of $CdCl_2$ concentrations on cadmium accumulation, dry mass production, photosynthetic pigment contents, TBARS content, antioxidative enzyme (CAT, SOD, APX, GPX and GR) activities and GSH/GSSG ratio in tomato plants. Results are the means ± SD of 6 replicates.

Parameter		Control	1 μM Cd	10 μM Cd	25 μM Cd	50 μM Cd
Cd content	roots	0	483 ± 9	1145 ± 120	1473 ± 189	1846 ± 179
[μg g^{-1}(DM)]	leaves	0	5 ± 0.9	9 ± 4	25 ± 8	49 ± 9
Dry mass	roots	13.6 ± 1.9	14.6 ± 2.5	12.4 ± 0.7	9.6 ± 0.4	8 ± 0.2
[mg]	leaves	295 ± 29	237 ± 24	102 ± 14	71 ± 13	64 ± 12
Pigments	Chl *a*	2.29 ± 0.08	1.68 ± 0.17	1.29 ± 0.13	0.80 ± 0.01	0.44 ± 0.12
[mg g^{-1}(DM)]	Chl *b*	1.02 ± 0.04	1.00 ± 0.04	0.75 ± 0.11	0.52 ± 0.07	0.52 ± 0.16
	Car	1.02 ± 0.07	0.92 ± 0.03	0.53 ± 0.12	0.26 ± 0.07	0.10 ± 0.07
TBARS	roots	29.65 ± 6	25.43 ± 10	52.77 ± 14	61.02 ± 12	64.36 ± 15
[mg g^{-1}(DM)]	leaves	75.43 ± 14	75.10 ± 12	142.63 ± 14	187.06 ± 20	266.70 ± 25
CAT	roots	0.71 ± 0.3	1.24 ± 0.5	2.15 ± 0.3	3.62 ± 0.4	4.30 ± 0.7
[mg g^{-1}(DM)]	leaves	13.93 ± 0.3	16.49 ± 1.0	12.22 ± 1.0	13.67 ± 1.6	12.82 ± 2.0
SOD	roots	1.30 ± 0.3	11.16 ± 2.6	14.64 ± 3.2	23.04 ± 6.0	37.85 ± 5.2
[mg g^{-1}(DM)]	leaves	6.22 ± 4.0	6.47 ± 6.4	12.07 ± 2.0	17.96 ± 3.6	22.50 ± 4.0
APX	roots	0.34 ± 0.09	0.78 ± 0.14	0.79 ± 0.12	0.62 ± 0.13	0.90 ± 0.20
[mg g^{-1}(DM)]	leaves	0.50 ± 0.03	0.17 ± 0.09	0.19 ± 0.09	0.48 ± 0.13	0.85 ± 0.20
GPX	roots	173 ± 39	659 ± 64	724 ± 82	838 ± 67	926 ± 81
[mg g^{-1}(DM)]	leaves	37 ± 9	16 ± 1.4	53 ± 12	353 ± 36	386 ± 4
GR	roots	0.05 ± 0.03	0.28 ± 0.09	0.37 ± 0.12	0.53 ± 0.14	0.54 ± 0.14
[mg g^{-1}(DM)]	leaves	0.19 ± 0.09	0.61 ± 0.10	0.62 ± 0.13	0.70 ± 0.12	0.95 ± 0.20
GSH/GSSG	roots	6.45	4.69	3.33	3.66	1.59
	leaves	12.02	7.75	7.22	4.37	4.73

In non-treated seedlings, CAT activity was higher in leaves than in roots. Cd treatment induced an increase of the CAT activity only in roots. In contrast to CAT, the total SOD activity was stimulated, in both organs. Cd also increased APX and GPX activities. In leaves as well as in roots, the highest activities were observed in plants treated with 50 µM $CdCl_2$ (Table 1). GR activity was higher in leaves than in roots of control seedlings and increased markedly in both organs of Cd treated ones.

In native PAGE, four SOD isoenzymes (bands I, II, III and V) were observed in control roots and leaves (Fig. 1). Cd-induced SOD activity seemed to be mainly due to the induction of a new SOD isoenzyme (band IV) in leaves and the increase in the intensities of four bands in roots. In non-treated plants, three and four APX isoenzymes were detected, respectively, in roots and leaves (Fig. 1). Cadmium induced accumulation of these isoforms in both organs. The non-denaturing PAGE gels stained for GPX activity revealed three and five GPX isoforms in leaves and roots, respectively (Fig. 1). There was a clear increase in the amount of GPX isozymes detected in Cd-treated organs as compared to the control ones. The GR activity, of tomato leaves and roots, following PAGE indicated that Cd treatment increased the intensity of the bands observed in control organs (Fig. 1).

The total glutathione content of both leaves and roots was affected by Cd treatment. A significant decrease in the GSH/GSSG ratios was observed in treated organs. Similar variation was the consequence of the decrease in the GSH content and the increase in the GSSG ones caused by Cd.

In tomato plants, Cd was mainly accumulated in roots and led to an inhibition of growth rate (Table 1). The decrease in leaves biomass production was especially observed in spite of the low metal concentrations. However, roots growth was damaged with the highest cadmium concentrations. The reduction in growth could be a consequence of the Cd-interference with a number of metabolic processes associated with normal development such as mineral nutrition (Drazic *et al.* 2006), membrane lipid composition (Ben Ammar *et al.* 2005) and photosynthetics pigments production (Table 1). The low sensitivity of roots to Cd could be explained by their capacity to accumulate the metal in a non-active form, fixed to the carboxyl (Clemens 2001) and/or sulphhydryl groups (Cobbett 2000).

The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in animals and plants (Cho and Seo 2005). The data obtained here suggested that cadmium induced an oxidative stress situation characterized by an accumulation of lipid peroxides (Table 1) and correlated with those announced by Dixit *et al*. (2001). Moreover, in a previous study, an alteration in membrane lipid composition has been observed in Cd treated tomato leaves. We have noted that Cd reduced the contents of chloroplastic and extrachloroplastic lipids. Likewise, levels of tri-unsaturated fatty acids: linolenic (C18:3) and hexadecatrienoïc (C16:3) dropped in membrane lipids (Ben Ammar *et al.* 2005).

Cd induced changes in detoxifying enzyme activities of tomato plants. In fact, Cd provoked a dose-dependent increase in SOD activity especially in roots (Table 1), which could represent a defence mechanism against Cd-induced $O_2^{\bullet -}$. Moreover, native PAGE analysis stained for SOD activity revealed that Cd induced of new isoform in leaves and increased the intensities of control bands in roots. Similar result suggested that SOD activity might play a crucial role in the response of tomato plants to metallic stress (Alsher *et al.* 2002; Fink and Scandalios 2002). Previous research examining the effects of Cd on SOD activity in plants produced various results, although the great majority showed a decrease or no change in SOD activity (Ferriera *et al.* 2002).

The inadequate response of CAT activity to Cd in leaves was compensated by an increase in the APX activity (Table 1). When APX was analysed by a native-PAGE a considerable stimulation in the intensity of all isoenzymes was detected (Fig. 1). CAT and APX were two potent scavengers of H_2O_2, their variations in leaves and roots suggested that they were functioning concurrently to remove H_2O_2 (Dixit *et al.* 2001).

In both organs studied, GPX activity (Table 1) and isoenzyme pattern (Fig. 1) were strongly affected by high concentrations of Cd^{2+}. Like in salinity stress, the GPX stimulation observed under Cd treatment may be caused by overexpression of genes coding for peroxidases or by activation of already synthesized enzyme isoforms (Mittal and Dubey, 1991).

In spite of the increase in the GR activity, a significant reduction in the GSH/GSSG ratio has been observed in Cd treated plants (Table 1), suggesting that a predominant GSH oxidation took place. De Pinto *et al.* (2000) indicated that the main functions of GSH in the protection against oxidative stress are its involvement in the ascorbate-glutathione cycle and in the regulation of protein thiol-disulphide redox status. The GSSG synthesized upon reduction of dehydroascorbate may be reduced by the glutathione reductase (Yu *et al.* 2002). Moreover, the decrease in GSH/GSSG ratio may be a result of the GSH incorporation into phytochelatins, observed in Cd-treated plants (Inhoue 2005).

In conclusion, our data indicated that Cd induced an oxidative stress in tomato plants, despite the concomitant increase in antioxidant enzymes. The accumulation of lipid peroxidation products indicated that the antioxidant system of tomato plants was not sufficiently effective in scavenging superoxide and peroxide radicals generated by Cd-stress.

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