

COMPETENCY OF ANTIOXIDANTS IN TOLERANT CULTIVAR OF EGYPTIAN CORN IN REDUCING OXIDATIVE INJURY CAUSED BY CADMIUM TOXICITY

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Abstract

Changes in growth, photosynthetic activity and antioxidative scavenging systems were investigated in leaves and roots of *Zea mays* L. seedlings treated with Cd²⁺ in order to investigate whether Cd²⁺ disturbs common plant defense pathways. Higher concentration of Cd²⁺ (100 µM) significantly reduced growth and leaf area of the seedlings. The contents of total chlorophyll (*a* + *b*) and carotenoids decreased, with a higher magnitude at high concentration of Cd²⁺. Furthermore, an alteration in activated oxygen species and increased lipid peroxidation was also detected. A significant decrease, especially with high concentrations of Cd²⁺, in the non-enzymatic antioxidant glutathione and GSH/GSSG ratio and enhanced accumulation of H₂O₂ were also observed. The activities of antioxidant enzymes i.e. superoxide dismutase, catalase, ascorbic peroxidase and guaiacol peroxidase rapidly enhanced in response to moderate concentration (20 µM), indicating the capacity of *Zea mays* L. plant to scavenge O₂⁻ and H₂O₂. A progressive increase of cell death in roots and accumulation of soluble phenolics were also concomitant with increase in Cd²⁺ concentration. The plant physiological stress indicators phenolics and carotenoids and the indicator of unbalanced metabolism of free radicals (concentration of MDA and H₂O₂) both were enhanced with respect to Cd²⁺ application.

Introduction

Environmental stresses that disturb the normal cellular metabolism can upset the balance of production and quenching of reactive oxygen species like superoxide radical, singlet oxygen and hydrogen peroxide. Cadmium is an increasing problem in soil due to industrial pollution, circulation of sewage sludge and use of commercial phosphorus fertilizers (Kumar *et al.* 2012). Cd²⁺ is one of the most toxic metals to vascular plants and considered biologically as a non-essential heavy metal. Although at low concentrations it was found to have stimulatory effect on growth of *Allium sativum*, at high concentrations it is extremely toxic to most plants (Liu *et al.* 2003). It has been proposed that cadmium can lead to oxidative stress resulting in inhibition of photosynthesis, respiration rate and other metabolic processes in plants (Hall 2002). Cadmium induces lipid peroxidation, as a result tissues suffered from oxidative stress leading to alterations in function of membrane (Chaoui *et al.* 1997, Cieccko *et al.* 2004). Photosynthesis is also sensitive to Cd²⁺ particularly the oxygen evolving reactions of PSII and also lowers the chlorophyll concentration in tomato plant before affecting photosynthesis (Baszynski *et al.* 1980 and Bavi *et al.* 2011). In this connection, Hassan *et al.* (2005) reported that Cd²⁺ decrease the biosynthesis of chlorophyll in barely through its effect on aminolaevulinic acid biosynthesis.

The involvement of antioxidants in plant responses against Cd²⁺ toxicity is unclear because Cd²⁺ does not belong to the group of transition metals like copper, zinc and iron which may induce oxidative stress via Fenton-type reactions. It is possible that the observed changes in the antioxidant systems occur, either as a result of unspecific cellular degradation process or that Cd²⁺

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triggers common defense pathways in plant cell like other biotic or abiotic environmental stresses (Bavi *et al.* 2011). One of the mechanisms involved in plant tolerance to Cd^{2+} is the synthesis of heavy metal binding ligand, phytochelatin (PC) from a reduced glutathione (GSH) in the cytosol for being sequestered into the vacuole (Vatamaniuk *et al.* 1999, Srivastava *et al.* 2004). The precursor of phytochelatin is reduced glutathione (GSH), whose cellular level decreased after exposure to Cd^{2+} (Xiang and Oliver 1998, Siroka *et al.* 2004). Glutathione is involved in the detoxification of heavy metals and xenobiotics and plays a role in gene activation and in the protection from oxidative stress (Gullner and Komives 2001). Besides an increase in the size of the glutathione pool, a high GSH/GSSG is necessary for the efficient removal of the reactive species generated under stress conditions. Thus, this suggests that glutathione is also involved in defense mechanisms against Cd^{2+} toxicity.

The ascorbate-glutathione cycle seems to be a mechanism of great importance in controlling the cellular redox status, especially after application of heavy metals (Tiryakioglu *et al.* 2006, Liu *et al.* 2007). An initial event of these pathways is an accumulation of H_2O_2 , which acts as a signaling molecule and induces an orchestrated sequence of reactions involving the activation of peroxidases, stimulation of secondary metabolism, and eventually cell death (Mishra *et al.* 2010).

The objectives of the present study were, therefore, to evaluate the response of maize to Cd^{2+} treatment and ascertain of some of the physiological reactions and the response of ascorbate-glutathione pathway to Cd^{2+} treatment in an effort to identify the possible involvement of Cd^{2+} in the generation of oxidative stress.

Materials and Methods

Seeds of maize from 10 cultivars were screened for their germination response to Cd^{2+} (data not shown). *Zea mays* L. cv. Giza 310 was the most tolerant variety. Seeds were surface sterilized in 0.1% HgCl_2 for 2 min; followed by five changes of sterile distilled water and soaked in aerated distilled water for 24 hrs in darkness. At the end of soaking period, 12 seeds were sown in each pot (15 cm diameter \times 20 cm height), filled with moistened vermiculite. All pots were placed in a growth chamber under 70 - 80% relative humidity with 16/8 hrs day/night cycle and controlled temperature of 28/26°C. Light intensity was 420 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by a mixture of fluorescent and incandescent lamps at the top of plants. Fifteen-days-old seedlings were carefully taken from the pots to avoid any injury to the roots and placed in sponge support collars. Collars were then fitted into holes in the tops of glass bottles containing 500 ml continuous aerated full strength Hoagland's solution. Cadmium was supplied as CdCl_2 at different concentrations (0, 10, 20, 50 and 100 μM); and pH of the cadmium solutions was adjusted to 6.0 and kept constant during the experiment with 0.1M phosphate buffer. Nutrient media were renewed every 12 hrs to maintain the metal concentrations. All bottles (five plants per bottle) were placed in a growth chamber under the same conditions. After two days of Cd^{2+} treatment, plants were harvested and washed carefully with distilled H_2O and blotted dry. Leaf area was determined using a moving belt electronic planimeter (Delta. T Devices, burwell, UK). Plants were dried in an air oven at 60°C until a constant weight to obtain dry weight.

Cadmium was determined as described by Verkleij *et al.* (1998) by an atomic absorption spectrophotometer (Perkin Elmer 2380) using an air-acetylene flame. Photosynthetic pigments *viz.* chlorophylls *a*, *b* and carotenoids were determined according to the method of Inskeep and Bloom (1985). 5- aminolevulinic acid (ALA) was extracted and estimated by the method of Stobart and Ameen-Bukhari (1984). The concentration of ALA was determined using the calculated extension coefficient 7.24×10^4 .

Malondialdehyde content was assayed by the method of Hodgson and Raison (1991). The extinction coefficient of 155 mM cm was measured by (Dai *et al.* 1997). Hydrogen peroxide was

extracted from roots in cold acetone for 6 hrs, and H_2O_2 was estimated as described by Patterson (1984). H_2O_2 level was calculated using the extinction coefficient $0.28 \mu\text{mol}/\text{cm}$.

Cell death, indicated as loss of plasma membrane integrity, was measured spectrophotometrically (Absorbance at 650 nm) by Evans blue uptake as described by Baker and Mock (1994). Free phenolic compounds were estimated according to Pritchard *et al.* (1997) using the Folin-reagent. Content of sulfhydryl (-SH) group was determined using the procedures described by Ellman (1959).

Measurement of GSH and GSSG content was carried out according to the enzymatic cycling method, using 5, 5 dithiobis (nitrobenzoic acid) DTNB and glutathione reductase (Anderson 1985). For the quantification of GSSG 2-vinyl pyridine was added to the extract to mask GSH. SOD activity was measured according to the method of Stewart and Bewely (1980). One unit of SOD activity was the amount of enzyme activity that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme. APX activity was assayed according to Asada (1992). One unit of APX was the amount of enzyme that oxidized $1 \mu\text{mol}$ of ascorbate per min at room temperature. CAT activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm (Aebi 1983). One unit of enzyme activity is equal to $1 \mu\text{mol}$ of H_2O_2 decomposed per min. GPX activity was determined according to Adam *et al.* (1995).

Total protein content of the extract used for the analysis was determined according to Bradford (1976) using bovine serum albumin as the standard. Data is reported as the mean \pm Sd **SD** of three independent replications. Samples from at least three plants were taken for each replication in all measurements. Results were examined by ANOVA. Means in individual experiments were tested for significance using least-significant-difference (LSD $p \leq 0.05$).

Results and Discussion

The toxic effect of Cd^{2+} on growth of shoot and root, shoot/root ratio, leaf area of maize was apparent at high concentration ($100 \mu\text{M}$) (Fig. 1). On the other hand, plants treated with low concentration ($10 \mu\text{M}$) showed slight but insignificant decrease ($p > 0.05$) in fresh and dry weights and leaf area relative to the control. It is interesting to note that the effect of Cd^{2+} was most exerted on root than shoot. As a consequence, the root/shoot ratio relative to the control decreased by about 47 and 55% at 50, and $100 \mu\text{M}$, respectively. At that time roots contained Cd^{2+} about 587 and 873 $\mu\text{g}/\text{g}$ dry weights, respectively, compared to 79 and 142 in leaves (Fig. 1A). The decrease in the fresh and dry weights of shoots and roots were closely related to the decrease recorded for the leaf area, which provoked a highly significant depression in the leaf area by 80 and 93%, respectively in the plants grown in Cd^{2+} concentrations 50 and $100 \mu\text{M}$ (Fig. 1C).

Exposure of plants to Cd^{2+} at 50 and $100 \mu\text{M}$ within 48 hrs resulted in a significant reduction in the length of roots and height of the shoot by about 53 and 59%, respectively (Fig. 1 B).

Cadmium treatment caused a general decrease in the total pigments content. At 50 and $100 \mu\text{M}$ Cd^{2+} , chlorophyll *a* content reduced to 76 and 91% and Chl *b* reduced to 69 and 84% respectively (Table 1). As a consequence, the Chl *a/b* ratio decreased from 3.2 (control) to 2.3 and 1.3 in leaves of the plant treated with 50 and $100 \mu\text{M}$ Cd^{2+} . There was significant reduction in carotenoids content, at 50 and $100 \mu\text{M}$ to less than 87 and 95%, respectively (Table 1).

Lipid peroxidation (determined as MDA level) significantly enhanced (4.7 and 7.4-folds) in roots of plants treated with 50 and $100 \mu\text{M}$ Cd^{2+} concentrations than corresponding control plants (Fig. 2B). Roots exposed to Cd^{2+} showed significant accumulation of H_2O_2 and browning of root tips with enhanced accumulation of soluble phenolics with increase Cd^{2+} concentrations compared with control (Fig. 2C). This resulted in decrease in the survival per cent, but did not lead to cell death (Fig. 2A). Evans blue uptake is an indicator of the loss of plasma membrane integrity and cell death (Baker and Mock 1994). After two days of Cd^{2+} treatment (50 and $100 \mu\text{M}$), maize roots exhibited high level of Evans blue uptake at 2.9 and 3.6 folds compared to control (Fig. 2D).

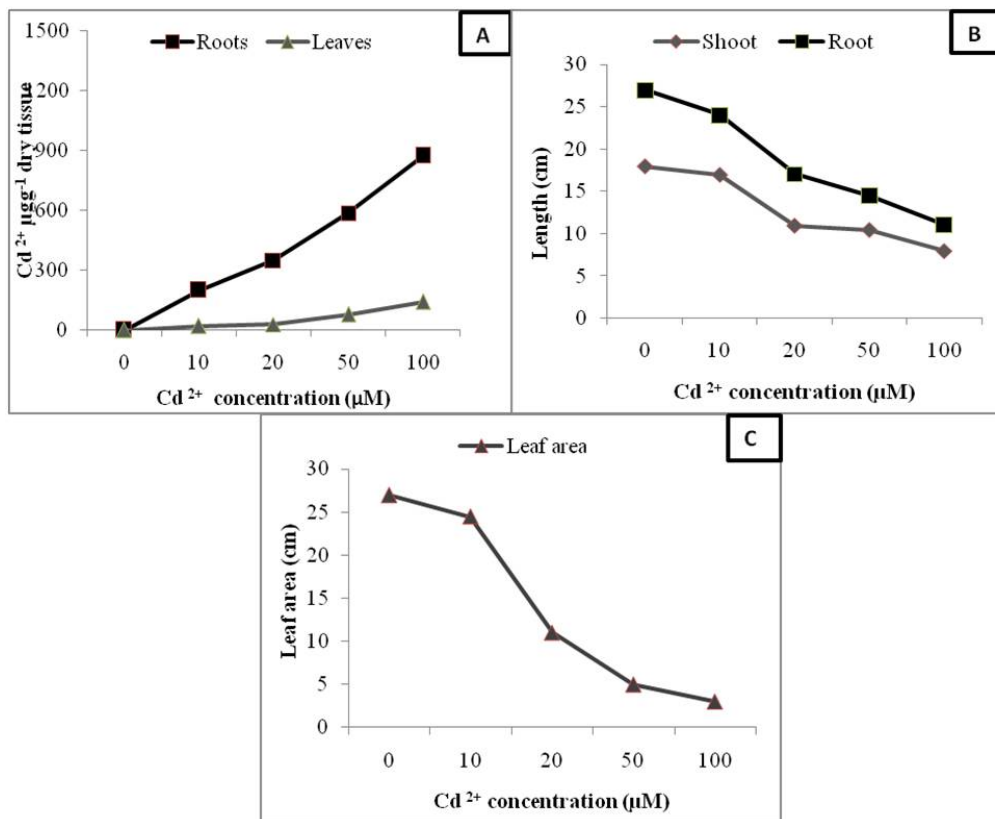


Fig. 1. Cd²⁺ accumulation in roots and leaves of maize seedlings treated with different concentrations of Cd²⁺ for 48 hrs (A). Effect of treatments are shown on the shoot and root length (B) and mean area of leaf (C). The results shown are means \pm SD of 3 replicates in (A) and 10 replicates in (B) and (C).

Table 1. Changes in Chl *a*, *b* contents; Chl *a/b* ratio and total carotenoids as well as percentage of 5-aminolaevulinic acid in leaves of maize plant treated with various conc. of Cd²⁺ for 48 hrs.

Pigment mg g/D.Wt.	Cd µM				
	Control	10	20	50	100
Chl <i>a</i>	7.01 \pm 0.8 ^a	4.54 \pm 0.4 ^b	3.63 \pm 0.4 ^b	1.53 \pm 0.2 ^c	0.45 \pm 0.1 ^d
Chl <i>b</i>	2.2 \pm 0.1 ^a	1.44 \pm 0.05 ^b	1.57 \pm 0.06 ^b	0.68 \pm 0.002 ^c	0.36 \pm 0.001 ^d
Carotenoids	1.81 \pm 0.2 ^a	1.74 \pm 0.1 ^a	0.7 \pm 0.03 ^b	0.24 \pm 0.005 ^c	0.09 \pm 0.003 ^d
Chl <i>a/b</i>	3.18 \pm 0.1 ^a	3.15 \pm 0.1 ^a	2.85 \pm 0.07 ^b	2.25 \pm 0.05 ^c	1.25 \pm 0.01 ^d
Aminolevulinic acid (%)	(100)	(87.4)	(39)	(13)	(7)

Values in parentheses were expressed as the per cent of reduction relative to the control (100%). Each value is the mean of three replicates \pm SD. Values carrying different letters are significantly different at $p \leq 0.05$.

The concentration of total SH-group in the roots after 48 hrs of Cd²⁺ treatment showed 160 and 240% enhancement at 10 and 20 µM Cd²⁺, respectively (Fig. 3C). Figure 3A also indicates that the amount of GSSG significantly increased, particularly at 50 µM Cd²⁺. The same trend was observed with GSH at concentrations up to 20 µM Cd²⁺, whereas at 50 and 100 µM Cd²⁺ these concentrations were lower than control. The fraction of GSSG increased significantly accounting 67% of total glutathione in the roots treated with 100 µM Cd²⁺. Cellular GSH/GSSG ratios were significantly decreased, particularly with the highest two Cd²⁺ concentrations (Fig. 3B).

There was significant increase of SOD activities except at 100 μM Cd^{2+} compared with control (Fig. 4A). CAT and APX activities were also significantly increased to some extent at 10 and 20 μM Cd^{2+} . At 50 and 100 μM Cd^{2+} , catalase activity was strongly decreased to lower than 37 and 16% , compared with the control; the corresponding values of ascorbic peroxidase activity were 17 and 5%, respectively (Fig. 4B and C). Guaiacol peroxidase activity greatly increased even at low Cd^{2+} concentration; a significant increase of 146% was found at 20 μM Cd^{2+} (Fig. 4D).

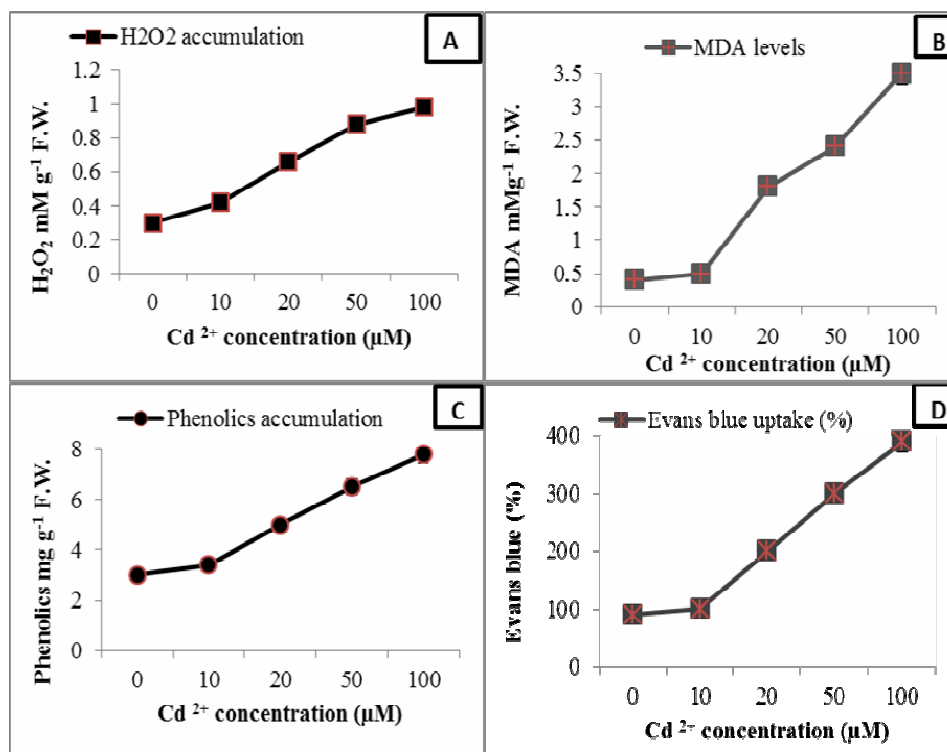


Fig. 2. Changes of concentrations of H_2O_2 , (A) MDA (B) phenolics (C) and cell death indicated as percentage of Evans blue uptake 48 hrs Cd^{2+} treated maize seedlings (D). Each value is the mean of three replicates $\pm\text{SD}$.

Other studies demonstrated that heavy metals can function as stressors, causing some physiological constraints that decrease plant vigour and inhibit plant growth (Ouzounidou 1994). The toxic effect of Cd^{2+} was apparent at its relatively high concentrations (50 and 100 μM), where it caused a significant decrease in shoot and root growth, leaf area and chlorophyll content of *Zea mays* plant. Inhibition of root growth within 48 hrs at 50 and 100 μM Cd^{2+} could be correlated with limitation of nutrients and water concomitant with Cd^{2+} accumulation (587 and 873 $\mu\text{g g}^{-1}$ D.wt.). This result was also confirmed by the work of Gussarsson *et al.* (1996).

The progressive decrease in leaf area (Fig. 1) and sharp decline in all photosynthetic pigment fractions (Table 1) and dry matter accumulation (Fig.1) with increase in Cd^{2+} concentrations might indicate an inhibitory effect on photosynthesis, via inhibition of chlorophyll biosynthesis (Ouzounidou 1994, Ouzounidou *et al.* 1995 and Hassan *et al.* 2005) or lipid peroxidation of the chloroplast membrane (Kunert and Foyer 1993). A significant decline was shown in chl *a/b* ratio especially with higher Cd^{2+} concentrations, which indicated faster breakdown or decreased synthesis of chl *a* as compared to chl *b*, since chl *b* was also decreased, a consistently reported

response to Cd^{2+} (Dub'e and Bornman 1992). However, changes in ALA content under elevated Cd^{2+} concentrations were significantly correlated with corresponding changes in their leaf chlorophyll content.

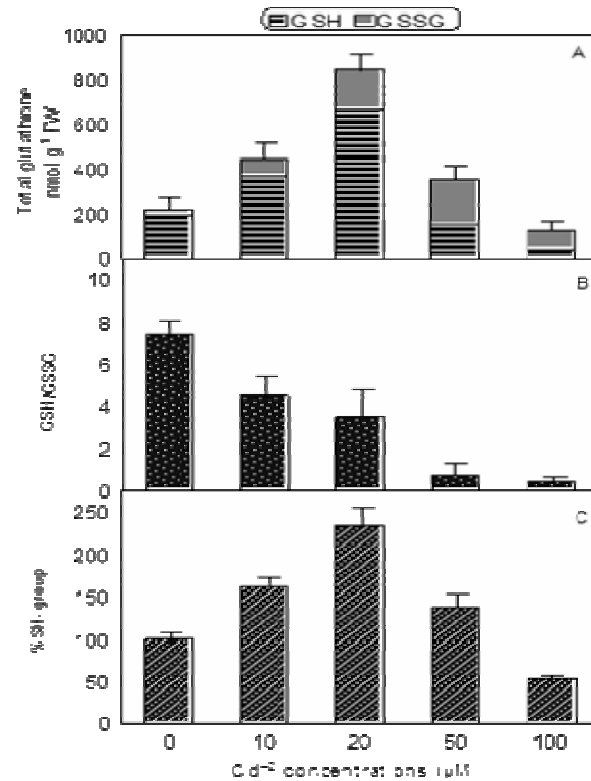


Fig. 3. (A) Glutathione accumulation, (B) GSH/GSSG ratio and (C) percentage of SH-group in roots of maize seedlings grown in the absence (0) or presence (10, 20, 50 and 100 μM) CdCl_2 . Each value is the mean of three replicates \pm SD.

Cadmium-treated roots showed more browning (phenolic-colored) than roots of control plants, which agreed with enhanced phenolic levels as shown in Fig. 2 i.e. Cd^{2+} caused rapid accumulation of soluble phenolics in the cytosol. Phenolics may contribute, together with ascorbate to H_2O_2 destruction in the so-called phenol-coupled APX reaction and thus protect cells from oxidative stress. In cultured tobacco cells, phenolics protect the living cell from aluminum toxicity (Yamamoto *et al.* 1998).

Cadmium shows affinity for sulfhydryl groups; reacts with hydroxyl groups and induces lipid peroxidation resulting in alterations in function of membranes. The cellular level of MDA represents a balance of oxidative stress-induced lipid peroxidation (Polle and Schützendübel 2003). Our results showed a marked progressive increase in MDA level with increase of Cd^{2+} concentrations (Fig. 2B). Stimulation of peroxidase activity and accumulation of H_2O_2 is a general stress response, which has been observed in plants exposed to different Cd^{2+} concentrations as reported for other stresses (Prasad *et al.* 1994). 50 and 100 μM cadmium-treated plants exhibited Evan's blue uptake at higher level than control, indicating the loss of plasmamembrane integrity and cell death. Similar results were obtained by Baker and Mock (1994).

As shown in the present study, higher GSH content was found during Cd^{2+} treatment (Fig 3A). The induction of GSH synthesis in plants is confirmed by the results of other authors in response to various abiotic stresses (Wingsle and Karpinski 1996, Ahmed *et al.* 2010). The ability to synthesize glutathione and availability of higher levels of GSH appear to be crucial for protection from cadmium (Pietrini *et al.* 2003). However, the amelioration of growth under cadmium stress by elevated GSH has not been observed in all cases, where exposure to heavy metals initially resulted in a severe depletion of GSH in many Cd^{2+} treated plants (Gullner and Komives 2001, Schutzendubel *et al.* 2001).

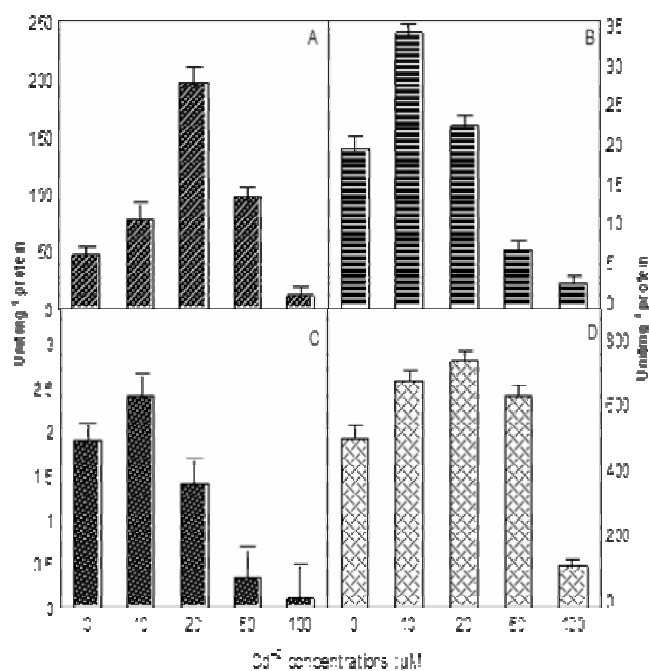


Fig. 4. Differential changes in antioxidant enzymes activities (A) SOD, (B) CAT, (C) APX and (D) GPX in roots of 15-day-maize seedlings exposed to different Cd^{2+} concentrations over a period of 48 hrs. Each value is mean of three replicates \pm SD.

In this study, the activities of antioxidant enzymes have been studied in roots in response to Cd^{2+} treatment. Since SOD enzyme controls the concentration of O_2 and its derivatives (OH^\cdot and H_2O_2), it is defined as a key antioxidant enzyme of plants (Adam *et al.* 1995). According to Savoure *et al.* (1999) Cd^{2+} interferes with SOD and also affects its isozymes differently. The results obtained showed that the total SOD activity in Cd^{2+} treated plants was stimulated to about 3.5 - folds that might result from a higher turnover of SOD a/o an increase of its inactivation by higher levels of H_2O_2 as an end product. APX plays an important role in scavenging H_2O_2 in chloroplast and in cytosol due to the presence of two APX isoforms with respect to its cellular localization (Meneguzzo *et al.* 1998). The present study showed that APX activity in comparison with control increased slightly with 10 μM Cd^{2+} . In contrast, high concentration of Cd^{2+} (100 μM) caused a significant decrease in its activity. Similarly, the CAT enzyme is also sensitive to O_2 and can be inactivated by its increasing levels resulted from Cd^{2+} treatment as discussed by Cakmak (2000).

A close relationship was observed between inhibition of root growth and development and the activities of four enzymes (SOD, CAT, APX and GPX) in roots. The plant physiological stress

indicators (concentration of phenolics and carotenoids) and the indicator of unbalance metabolism of free radicals (concentration of MDA and H₂O₂) both were enhanced with respect to Cd²⁺ application.

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