Egypt. J. Bot. 43, No. 1-2, pp.43-54 (2003)

Oxidative Damage and Free Radical Formation in Maize Seedlings after Exposure to Cadmium Stress

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FOR better understanding of the different biochemical processes caused by cadmium stress, maize plants (*Zea mays* Lcv single cross – 10) were grown in a complete nutrient solution with addition of 25μM or 50μM Cd as Cd $(NO_3)_2$ for 15 days. Inhibition of plant growth is the most sensitive response to Cd stress. The accumulation of Cd in roots and leaves increased with increasing external Cd concentration in the growth media and was considerably higher in roots than in leaves. Excess Cd also reduced chlorophyll content in leaves.

Furthermore, substantial increase of antioxidant enzymes and antioxidant compounds such as peroxidase (POX), glutathione S-transferase (GST) and glutathione (GSH) respectively were observed in Cd stressed plants in comparison with control plants. An enhanced level of lipid peroxidation in root tissue indicated that Cd caused oxidative stress in maize plant. These differential responses to Cd were further found to be associated with levels of Cd to which the plants were exposed. The protein profile of root tissue obtained by SDS- PAGE technique showed new band with molecular weight ≃ 84 KD under 50μM Cd compared to control treatment.

The level of free radial formation in root tissue (the first organ exposed to Cd toxicity) was measured by using electron spin resonance (ESR) method. The results showed that at the 25 μM or 50 μM Cd, the lines of ESR signal were similar but still higher compared to that of control treatment. The detection of such parameters is useful, early and sensitive indicators for prediction of heavy metal toxicity.

Keywords: Cadmium stress, glutathione, lipid peroxidation, protein electrophoresis, electron spin resonance, and maize.

Cadmium is a heavy metal that is toxic for humans and plants, however plants like all other organisms have to tightly control the intercellular concentration of such non- essential toxic heavy metal. Increasing level of toxic heavy metals (due to industrial pollution and the use of commercial phosphorous fertilizers) affects various physiological and biochemical processes in plants (Vangronsveld and Clijsters, 1994). This leads to a reduction of growth due to inhibition of

chlorophyll synthesis and photosynthesis (Stobart *et al.*, 1985). One of the consequences of the presence of toxic metals in plant cells is the formation of free radical species such as $O_2\cdot^-$, $OH^\cdot$, H_2O_2 that can cause severe damage to different cell components (Referred as oxidative stress) including proteins, membrane lipids and nucleic acids (Dixit *et al.*, 2001). Plants have developed various mechanisms for the protection from oxidative stress. The antioxidative system of plants comprises several enzymes (CAT, POX, SOD) and non enzymatic compounds (Vit. C, Vit. E, GSH) that are principally constitutive and vary in plants at cellular level (Cho and Park 2000). SOD, the first enzyme in the detoxifying process, catalyzes the dismutation of $O_2\cdot^-$ to H_2O_2 and O_2, then the POX reduces H_2O_2 to H_2O using several reductions available in the cells (Foyer *et al.*, 1994). Altered activities of these antioxidant systems commonly have been reported in plants and are used frequently as indicators of stress (Koricheva *et al.*, 1997). Also, Cd stress induced modification in protein subunit, which displayed with one dimensional gel electrophoresis analysis (Ramirez *et al.*, 1999). In addition heavy metals stress cause direct effect on electron transport pathway in mitochondria and chloroplasts. (Elstner and Osswald, 1994). The ESR techniques have been implicated to detect the formation of free radicals in plants during nutrient stress (Cakmak and Marschner 1988). The ESR method can also be utilized for estimating the type and content of more stable free radicals that accumulated in cells as markers of oxidative stress due to O_2 enrichment (Goodman *et al.*, 1986). The objective of the present study was to determine whether such biochemical changes (some antioxidant enzymes, lipid peroxidation, protein profile, ESR) could be used as indicators for prediction of metal effects and toxicity.

Material and Methods

Plant material and growth condition

Grains of maize (*Zea mays* L. *cv* single cross -10) were sown on filter paper saturated with distilled water and incubated at 26°C in the dark. Three days later, seedlings selected for uniform growth were transplanted into 2-L tanks (10 seedlings per tank) containing an aerated complete nutrient solution with the following composition: [200 µM KNO_3, 200 µM $Ca(NO_3)_2$, 40 µM KH_2PO_4, 200 µM $MgSO_4$, 25 µM Fe-EDTA 30 µM H_3BO_4, 5 µM $MnCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$, and 0.1 µM $(NH_4)_6Mo_7O_{24}$, pH 6.50] and kept in at 26 -22°C and 70% relative humidity during the 14/10-h light/dark period . The complete nutrient solution was subjected to three Cd treatments, (1) no cadmium supply (control) expect by way of water and essential nutrient salts, (2 , 3) 25 or 50 µM Cd as $Cd(NO_3)_2$. Hydroponics solutions were renewed every two days to minimize nutrient depletion. At appropriate time (15 days growth period), whole plant samples were removed and used for the following determinations.

Cadmium determination

Plants were harvested (roots, leaves) then washed for 10 min in ice-cold 5 mM $CaCl_2$ solution to displace extracellular. Plant tissues were excised and

gently blotted with paper towels. After the wet digestion in a mixture of nitric, sulfuric, and perchloric acid (5:1:1, v/v), Cd content was measured by atomic absorption spectrophotometer (Varian SpectrAA).

Chlorophylls determination

The chlorophyll contents of new leaves was determined after extraction by 80% acetone. The absorbance of the extract was measured using spectrophotometer at 663 nm and 646 nm according to Lichenthaler (1987).

Enzymes determination

Protein extraction

Soluble protein extracts were prepared at 4C° from Cd-treated and un-treated maize plant. Fresh leaves (usually 0.5 g) were homogenized in ice-cold 250 mM sucrose buffer (pH 7.2) in a chilled pestle and mortar. The homogenate was centrifuged at 12.500 rpm for 20 min at 4C°. The supernatant obtained after centrifugation was used for measurement of enzymes activity. Proteins in the extracts were quantified by the method of Bradford (1976) in order to calculate the specific activity of the enzymes.

Peroxidase activity

(EC 1. 11. 1.7) was determined by spectrophotometry method according to Amako *et al.*, (1994). The reaction mixture contained 1.5 ml of 100 mM K-phosphate buffer (pH 6.8),1 ml of 60 mM pyrogaloll, 0.48 ml of 0.6 mM hydrogen peroxide and 20µl of the enzyme extract. Monitoring the increase in the absorbance at430 nm followed oxidation of pyrogaloll.

Glutathione s- transferase

(E.C 2.5.1.18) (GST) activity was assayed in a reaction mixture containing 50mM phosphate buffer, pH 7.5, 1 mM of 1-chloro- 2, 4 dinitrobenzene (CDNB) and crude extract equivalent to 100 µg of protein. The reaction was initiated by the addition of 2 mM GSH and formation of S- (2,4-dinitro phenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm according to Li *et al.* (1995).

Glutathione determination

The level of total acid-soluble SH compound (glutathione) was determined with Ellman's reagent according to Tukendorf and Rauser (1990). The absorbance at 412 nm was read after 2 min (Σ=13.6 mM^{-1} cm^{-1}).

Lipid peroxidation (TBA test)

The level of lipid peroxidation products in the roots was assayed using 2-thiobarbituric acid reagent (TBA) according to Buege and Aust (1978). The absorbance of the end product of lipid peroxidation (mainly malondialdehyde, MDA) was measure at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The level of the lipid peroxidation products was expressed as nmol MDA / mg pro. / hr. (Σ= 1.56×10^5 M^{-1} cm^{-1}).

Protein electrophoresis

Protein extract of leaves were identified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using 12% (w/v) polyacrylamide and 0.1% (w/v) SDS. Each sample (30 µg protein) was loaded onto the slots. The gel was stained with coomassie blue R 250, and destined with an aqueous acetic acid and methanol solution. Molecular weight marker (Sigma) ranging from 26 to 180 K.D, to estimate molecular weight of the purified protein.

The spectra of electron spin resonance (ESR)

Representative sample composed of 10 roots from each treatment were excised on ice and stored at –20°C. Frozen samples were lyophilized, and were then ground in a mortar. The powdered material (500 mg) was packed into ESR flat quartz cuvette. The ESR spectra were recorded at room temperature using ESR spectrometer ELEXSYS E500 (Bruker-Germany).

The standardization of "g" value was carried out using 1,1 Diphenyl –2-picryl-hydrazyl (DPPH) as a standard. The areas under ESR curve for each sample were calculated. The area under the curve of the control sample was represented as 100% and was used to plot the relative radicals versus Cd concentration. The data presented here are from repeated at least twice experiment, the mean value ± S.E are reported in tables.

Results and Discusion

1- The plant growth

The earliest symptom of Cd toxicity in seedling was exhibited after four days growth. The stunted growth of leaves and roots is shown in Fig . (1). In addition, the presence of 50 µM Cd in the growth media showed visible effects on root, shoot morphology concomitantly, plants developed short lateral roots and marginal chlorosis of the younger leaves. This inhibition might be the result of disturbances in cell division or the toxic effects of Cd, which drastically led to cell death. This is in accordance with the results obtained by Vitoria *et al.,* (2001).

2- Cd accumulation and chlorophyll content

As seen from Table (1) most of Cd that entered plant system accumulated in the root and only a small amount was translocated to the leaves. Cd accumulation being approximately 5 times higher in roots than in leaves,which probably indicates an efficient Cd exclusion at the root surface.

Also, the total Cd concentration of roots and leaves in maize plants increased concurrently with the treatments applied. Maize plants might be considered as Cd-shoot excluders (Florijn and Van- Beusichem 1993), since Cd accumulated at higher concentrations in roots than in leaves. This behaviour is one of several strategies of tolerance to Cd toxicity (Weigel and Jager, 1980). Cadmium retention in root might be due to linking of Cd to carboxyl groups of the cell wall (Barcelo and Poschenrieder, 1990).

c b a

Fig. 1. Plants growth as affected by different cadmium concentrations after 4 days in nutrient solutions.
a- Control
b - 25 µM Cd
c-50 µM Cd

TABLE 1. Accumulation of Cd in roots and leaves and chlorophyll contents in leaves of maize seedling grown for 15 days with 25 µM and 50 µM of Cd.

Treatment	Cd content (µg/g D.W)		Chlorophyll content (mg/g F.W)	
	Root	Leaves	a	b
Control	3.0 ± 0.2	0.9 ± 0.1	0.86 ± 0.19	0.29 ± 0.12
25 µM Cd	70.5 ± 2.1	14.2 ± 1.7	0.67 ± 0.14	0.19 ± 0.13
50 µM Cd	116 ± 2.3	24.0 ± 1.9	0.65 ± 0.11	0.15 ± 0.11

Addition of 25 or 50 μM to the growth medium reduced the content of chlorophyll a and b in maize leaves. When chlorophyll a and b contents were measured separately, greater loss of chl. b than chl. a under 50 μM Cd treatment was observed. One possible explanation for the Cd- induced reduction of chlorophyll is that Cd damages the structure of chloroplasts (Ouzounidou *et al.*, 1997). Heavy metals are known to inhibit the activities of 5- amino – levulinic – acid dehydratase and chlorophyllase, enzymes required for synthesis of photosynthetic pigments. (Prassad and Prassad, 1987).

3- Enzymes activity

The level of enzymes activity in roots and leaves of maize seedlings subjected to Cd stress is presented in Table (2). Generally POX and GST activities were lowered in roots than in leaves of Cd-treated and un-treated maize seedling. It was found that, Cd- induced an increase in the activities of POX, which was higher in leaves (116.8% and 111.2% of the control at 25μM and 50μM, respectively). Whereas GST activity in leaves of plants exposed to 25 μM or 50 μM Cd progressively increased to 136% and 216% of the control, respectively. In addition GSH, that maintains the cellular redox status, showed a concentration decrease in its level in Cd- exposed plants (Table 2). As compared to the control, the decrease was more pronounced in roots (50% at 25 μM Cd and 34% at 50 μM Cd) than in leaves (725% at 25 μM and 67.8%at 50μM Cd).

TABLE 2. Enzymatic activities, glutatione content in leaves and roots and MDA content in roots of maize seedlings exposed for 15 days to 25 μM or 50 μM of Cd.

Treatment	POX activity ± S.E. (% of control)		GST activity ± S.E. (% of control)		GSH content ± S.E. (% of control)		MDA content ± S.E (% of control)
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Roots
Control	12.5 ± 0.30 (100)	2.66 ± 0.12 (100)	025± 041 (100)	019±031 (100)	1858±021 (100)	358±015 (100)	151±2.1 (100)
25 μM Cd	14.6 ± 0.37 (116.8)	4.9 ± 0.25 (184.2)	034±051 (136)	021±043 (1105)	1348±023 (725)	1.79±032 (50)	353±37 (2338)
50 μM Cd	13.9 ± 0.32 (111.2)	3.8 ± 0.14 (146.1)	054±021 (216)	030±070 (1578)	126±041 (678)	123±051 (3435)	535±25 (3543)

POX activities were expressed in unit/ mg Protein.
GST activities were expressed in unit/ mg Protein.
GSH contents were expressed in μ mol/g F.W.
MDA contents were expressed in nmol/ g F.W.

The presence of Cd in the nutrient solution caused an evident increase in some antioxidant enzymes, which considered playing an impotent role in the cellular defense strategy against oxidative stress (Van Asche and Clijsters, 1990). Increasing activity of POX activity might be related to the lower H_2O_2 production, which resulted from oxygen free radicals formation. High activities of GST explain the fact that, GST is able to metabolize toxic exogenous products of lipid peroxidation and DNA damage in maize plant (Marrs and Walbot, 1997) as well as to keep non-essential metal (Cd) at low toxicity level.

The depletion of GSH content may be due to its conjugation with xenobiotic substance that is an essential antioxidatant considered as a precursor for phytochelations compound synthesis (Dixit *et al.,* 2001). Addition of Cd to tissue culture cells was accompanied by a fall in the concentration of gluathione, this phenomenon could be explained as follows; Cd may be detoxified by combination with peptides termed phytochelations. These peptides are structurally related to glutathione (Vitoria *et al.,* 2001). When a stress occurred, plants activated stress coping mechanisms (repair processes, antioxidant, *etc.*,), which led to a hardening of plants. Then after reaching a maximum of resistance, a too acute stress overloaded cellular defense of plant which vitality then steadily decreased.

The TBA assay can be regarded as a reliable method for evaluating the degree of lipid peroxidation. The results, as MDA content on basis of fresh weight are shown in Table (2). Compared to control, concentration of MDA increased by the increase of Cd treatment indicating enhanced lipid peroxidation in metal exposed root organ. The observed changes in the MDA content were consistent with previous results observed by Dixit *et al.,* (2001) these authors stated that, an enhanced level of lipid peroxidation of pea plants exposed to Cd indicated that the metal caused oxidative damage to plants. It means that Lipid peroxidation may be a consequence of generation of reactive oxygen species ($OH^{\cdot}$, $O_2^{\cdot -}$, H_2O_2).

4- Protein electrophoresis

The distributions of total polymorphic bands of protein of maize roots grown under cadmium stress are photographed as shown in Fig . (2). It was found that, the electophoretic pattern of all treatments showed three distinct regions depending upon the protein molecular weight, *i.e.* high proteins (> 100 KD), medium proteins (<60 KD) and low molecular weight proteins (< 20 KD). Meanwhile, slight differences in the protein patterns between treated and untreated plants were observed. High cadmium concentration (50 µM Cd) which presented in lane no 3 showed one new band with high molecular weight ($\simeq$ 84 KD). Thus, this may be related to some mechanisms in root tissue that can synthesize new protein in order to regulate Cd toxic effect. This information may be in agreement with the findings of Ramirez *et al.,* (1999) these authors mentioned that under copper toxicity increases in certain protein bands, detected by SDS-PAGE method were observed. These new protein bands could be related to Cu (II) uptake. This fact could be used as indicator of heavy metal contamination.

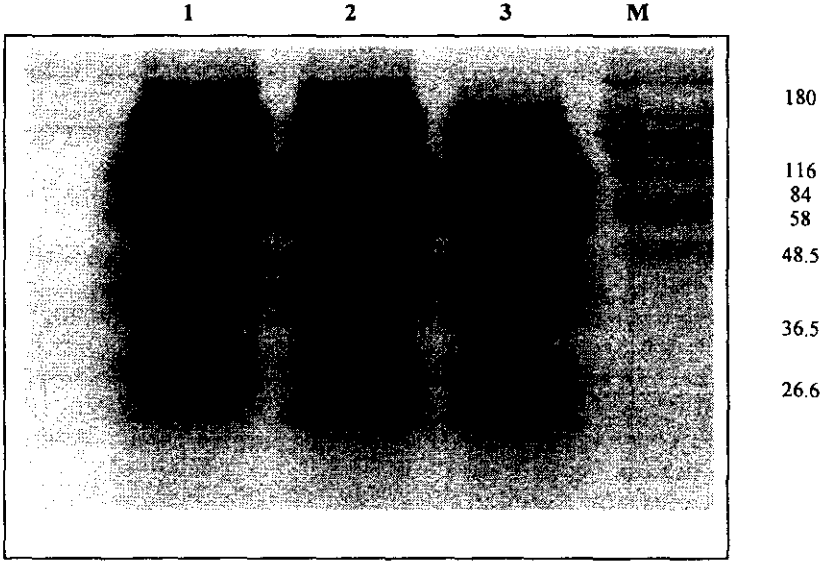

Fig. 2. Protein profiles of roots of maize seedling subjected to Cd stress:
Lane 1 - Control plant
Lane 2 - 25 µM Cd
Lane 3 - 50 µM Cd
Lane 4 - Protein marker with molecular weight 26.6-180 .

5- Spectra of ESR

The free radical productions under Cd stress in root tissue were detected with ESR and the spectra of ESR signal was illustrated in Fig. (3). Under control conditions, the spectrum area was lower than that in treated samples (g = 18.7, considered as 100%). At 25µM of Cd, the amount of free radical was similar to 50µM (g = 33.6 and 37.1 respectively, which represent 179.6% and 198.4% of the control). The intensity, which related to free radical formations, may correspond to the stable free radicals airside from the reaction of superoxide free radical with one or more of the constituents of the root cells such as DNA, lipids and proteins.

A relationship between Cd toxicity and the concentration of free radicals has been revealed. Therefore evaluation of free radicals in the root tissue can be used as a marker for oxidative damage caused by Cd exposure which caused formation of superoxide anion in plant cells (Vangronsveld and Clijsterd, 1994). In conclusion Cd toxicity negatively affected the plant growth and activities, which consequently will reduce the plant productivity.

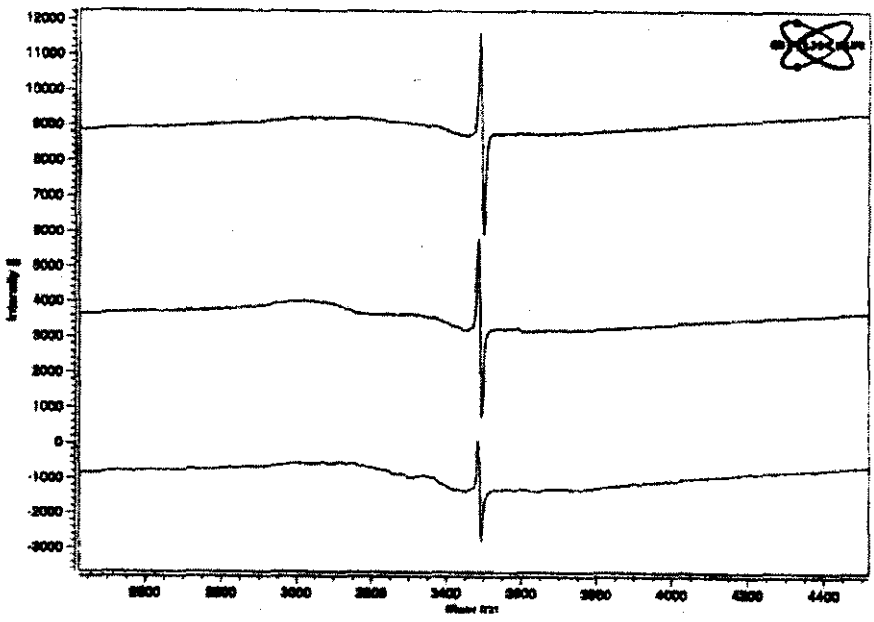

Fig .3. The signals of free radical formation in roots tissues of control (bottom) and of roots treated for 15 days with 25 µM Cd (middle) or with 50 µM Cd (top).

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(*Received* 31 / 12 / 2003;
Accepted 30 / 3 / 2004)

التلف التأكسدى وتكوين الشقوق الحرة فى بـادرات الـذرة بعـد التعرض للكادميوم

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لإمكانية الفهم الجيد للعمليات الكيميائية الحيوية التي تحدث للنبات عند التعرض لعنصـر الكادميوم (فى صورة نترات كادميوم) فى نبات الذرة (صنف هجين فردى ١٠) تم تنميـة النبات فى محلول مغذى (مزارع مائية) فى وجود ثلاثة معاملات هي كالآتي: –

١– ظابط

٢– ٢٥ ميكرومول كادميوم

٣– ٥٠ ميكرومول و استمرت التجربة لمدة ١٥ يوما وقد حدث تثبيط لنمو النبات.

وكانت النتائج المتحصل عليها كالآتي: –

١– أدت زيادة كمية الكادميوم فى المحلول المغذى إلى زيادة تراكم عنصر الكادميوم فى كل من الجذور والأوراق ولكن الزيادة فى الجذور كانت أعلى.

٢– نقص فى كمية الكلوروفيل بزيادة تركيز عنصر الكادميوم.

٣– حدثت زيادة فى الأنزيمات المضادة للأكسدة مثال البيروكسيديز والجلوتاثيون – S– ترانسفيرز وكذلك زيادة فى مادة الجلوتاثيون فى حالة المعاملة بالكـادميوم بالعينـة الظابطة.

وهذه الزيادة كانت دائما فى الأوراق أكثر منها فى الجذور.

٤– حدثت زيادة فى أكسدة الدهون وذلك فى أنسجة الجذور بزيادة التعرض للكادميوم.

٥– حدث تغيير فى وحدات البروتين المفصولة بواسطة تقنية الألكتروفوريسيس SDS- PAGE وظهرت حزمة وزنها ٨٤ دالتون كيلو.

٦– تم تقدير كمية الشقوق الحرة المتواجدة فى الجذور بواسطة جهاز التـردد المغزلـى الإلكتروني (ESR) .

وجد تشابه فى إشارات الجهاز فى حالة المعاملة بعنصر الكادميوم عند تركيز ٢٥ أو ٥٠ ميكرومول وكانت هذه الإشارات عالية عن المقدرة فى المجموعة الضابطة الكنترول.

التقديرات السابقة من الممكن اعتبارها تقديرات سريعة – حساسـة للتنبـأ بسـمية العناصر الثقيلة.