

## Oxidative Stress and Antioxidant Defense Mechanisms in Response to Cadmium Treatments

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**Abstract:** Effect of Cd treatments on the changes of fresh and dry weights, leaf area, root length and shoot height were studied at 10, 50 and 100  $\mu\text{M}$  of Cd. It was found that fresh and dry weight of both shoot and root, shoot height, root length and leaf area were significantly reduced and the deleterious effect of Cd on sunflower plant became more severe with increasing Cd level and extended time of exposure. The results also showed that the total chlorophyll content was reduced. This reduction was less pronounced in chlorophyll *b* than in chlorophyll *a*. Accordingly, the ratio of chlorophyll *a* to chlorophyll *b* tended to decrease with increasing Cd concentration. At the same time the total carotenoids content was increased at the concentrations of 10  $\mu\text{M}$  Cd. Also a significant alteration in elements status was observed. In order to investigate whether Cd induces common plant defense pathways, the temporal sequence of different physiological and biochemical reactions including lipid peroxidation, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production and the activities of some antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), in addition to the changes in ascorbate-glutathione-related antioxidant systems were studied in both leaves and roots of sunflower plant to know the possible involvement of Cd metal in the generation of oxidative stress. It was found that, oxidative stress in sunflower plants were took place at the different concentrations of Cd ions and with increasing of exposure time as evidenced by an increase in lipid peroxidation and electrolyte leakage of elements. Antioxidant enzymes activities were also stimulated by Cd treatment and was associated with changes in ascorbate and glutathione level with a larger pool of dehydroascorbate (DHA) and reduced glutathione contents (GSH) suggesting that Cd can be added to the list of stresses such as drought, heat shock, ozone and  $\text{SO}_2$ . Finally, the obtained results in this study suggest that growth of sunflower plants in the presence of Cd metal showed a concentration-dependent oxidative stress situation in both leaves and roots as a result of the inhibition of the antioxidant systems.

**Key words:** Antioxidant systems % Cadmium % Oxidative stress % Lipid peroxidation % Electrolyte leakage % Sunflower (*Helianthus annuus* L.)

### INTRODUCTION

The unprecedented, rapid change in environmental conditions is likely to override the adaptive potential of plants. Heavy metals contamination caused by either natural processes or by various industrial wastes in addition the diverse human activities such as metal-working industries, mining and smelting and the uses of mineral fertilizers as well as pesticides or from other sources [1] are the most serious environmental problems.

Among the pollution-producing metals, cadmium (Cd) is a widespread heavy metal in the environment and it is regarded as non-essential elements and have a long half-life which is extremely persistent in the environment

[2, 3], with high toxicity and easily taken up by plants [4] and then enters the food chain, resulting in a serious health issue for animals and humans. Therefore, there is an increasing interest in effects of heavy metals on higher plants and their responses to excessive metal concentrations as stressors [5].

Although, cadmium has no known a biological or physiological function, so plants can accumulate cadmium ions to various extents and, in some cases, these ions reach levels that may be toxic for plant [6]. Consequently, it can induce a number of deleterious effects at morphological level such as reduction in the shoot height and root length and changes in the color of the root from yellowish to browning, also changes in the

biochemical and physiological processes that lead to cellular damage [7, 8, 9,10] and damage to plasma membrane permeability by triggering strong lipid peroxidation [11-13]. Furthermore, it was found that Cd often impairs photosynthesis at several physiological levels, stomatal conductance, pigments content, chloroplast structure and function [14].

The presence of cadmium has also been associated with the appearance of oxidative stress [15] and it is also assumed that it directly or indirectly causes the formation of active oxygen species (AOS) [16], thus interfering with the redox status and inducing significant chlorophyll loss [17,18]. Therefore, the accumulation of reactive oxygen species (ROS) due to the presence of Cd, can damage different cell structure and function through oxidation of several macro-molecules [19].

However, it is well established that plants, as soon as any heavy metal penetrates the roots or the leaves, stimulate the efficiency of antioxidative endogenous defense mechanisms that can remove, neutralize or scavenge oxygen radicals and their intermediates in the cells by antioxidant enzymes such as superoxide dismutase (SOD: EC.1.15.1.1) for removal of  $O_2^-$ , catalase (CAT: EC.1.11.1.6), ascorbate peroxidase (APX: EC.1.11.1.11) for removal of  $H_2O_2$  and glutathione reductase (GR:EC.1.6.4.2). For example, in roots and leaves of Cd-exposed *Phaseolus vulgaris* as well as suspension cultures of tobacco (*Nicotiana tabacum*) cells contained elevated APX activities after Cd exposure [12,20]. In *Phaseolus aureus* seedlings, Cd induced elevated guaiacol peroxidase (POD) but decreased CAT activities [21]. In addition to these antioxidant enzymatic systems, the plants contain non-enzymatic antioxidant systems involving substances like  $\beta$ -carotene,  $\alpha$ -tocopherol and reduced form of ascorbic acid and glutathione. A relevant defense system may be represented by glutathione, which protects many cellular components against oxidative stress.

To understand how the presence of Cd ion can affect growth, physiological and biochemical processes within the plants might bring new solutions to the problems raised by heavy metals contamination. Thus, the objective of the present study is directed to investigate the sequence of some physiological and biochemical changes, regarding chlorophyll content in leaves, metal uptake and translocation,  $H_2O_2$  production, lipid peroxidation and electrolyte ions leakage. Also, the response of some antioxidant enzymes such as SOD, CAT, APX and GR are involved and ascorbate-

glutathione pathway to Cd treatment was investigated and their roles in scavenging active oxygen species are discussed in an effort to identify the mechanism(s) by which Cd decreases plant growth and developmental changes occurring in leaves and roots of sunflower (*Helianthus annuus* L.c.v. Vedoc hybrid plants) as one of the most important economic oily crops in Egypt.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions:** Seeds of sunflower (*Helianthus annuus* L.c.v. Vedoc hybrid) were surface-sterilized for 30 min in 5% (v/v) commercial bleach. After rinsing several times with distilled water, seeds were soaked in aerated distilled water for 24 h, then allowed to germinate on moist filter paper placed in sterilized Petri dishes for 3 days in complete darkness at 28°C. The homogenous seedlings were chosen and carefully transferred to growth jars (10 cm diameter  $\times$  25 cm height) containing a continuously aerated full strength Hoagland's solution [22] which was covered with a polystyrol-plate with 5 evenly spaced holes. One seedling was fixed in each hole and the growth jars were placed in a growth chamber under 16 h photoperiod 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photo synthetically active radiation (PAR). Light was provided by a mixture of fluorescent and tungsten lamps at the top of plant. The average temperature was 25°C/20°C (day/night) and 70%-80% relative humidity. The nutrients were replaced twice a week. Air was continuously and gently bubbled through all jars. After 2 weeks Cd was added as  $\text{CdSO}_4$  to the nutrient solutions to give concentrations of 0, 10, 50, or 100  $\mu\text{M}$  Cd. pH of the solution was adjusted to  $6.5 \pm 0.1$  with NaOH or HCl as required.

At the desired time of each Cd treatment, plants were harvested and washed carefully with  $H_2O$ , blotted dry and separated into roots, stems and leaves. Leaf area was determined using a moving belt electronic planimeter (Delta. T. Devices, burwell, UK). Fresh weights of different parts were determined and then the same parts were dried in an air oven at 70°C until a constant weight to obtain dry weight.

**Measurements of Photosynthetic Pigments:** Photosynthetic pigments, viz, chlorophyll *a*, chlorophyll *b* and carotenoids were extracted and determined from expanded young leaves according to Inskeep and Bloom [23]. Known fresh weight (about 0.1 g) of leaves were immersed in 10 ml N, N-dimethylformamide (DMF) and

kept overnight at 4°C. After incubation, chlorophylls contents (Chl *a* and Chl *b*) and total carotenoids were determined in the extract by UV-spectrophotometer (LKB, UK). The absorbance of the solution was measured between 400 and 700-nm.

**Macronutrient and Micronutrient Determination:** The dry samples of shoot or root were finely ground and assayed for mineral-ions contents according to the method described by Humphries [24]. Metal concentrations ( $\mu\text{g g}^{-1}$  DW) such as Fe, Mn, Mg and N were estimated by atomic absorption spectrometry and Ca, Na and K by flame photometry. The values were expressed as  $\mu\text{g g}^{-1}$  dry weight of root or leaf for each treatment. Electrolyte leakage was measured according to the method described by Humphries [24].

**Cd Content Determination:** Determination of Cd was carried out on leaves and roots of sunflower plants. After 24 h intervals of Cd treatment, leaf and root samples were dried at 80°C for 48 h and the dry weight was measured. Determinations of metals were made by atomic absorption spectrophotometry (Analyst 300; Perkin Elmer, Germany) on nitric-perchloric acid (3:1, v/v) digests of four replicate samples from plant tissue according to Rauser [25].

**Lipid Peroxidation Determination:** Malondialdehyde (MDA) content as indicator of the level lipid peroxidation of plasma membrane of plant cells of tissue was estimated by the method of Dai *et al.* [26]. The level of lipid peroxidation is expressed as  $\mu\text{mol}$  of MDA formed using an extinction coefficient of 155  $\text{mM}^{-1}\text{cm}^{-1}$ .

**Determination of H<sub>2</sub>O<sub>2</sub> Levels:** Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was extracted according to Vivek *et al.* [27] and measured following the method of Okuda *et al.* [28]. The reaction was initiated by the addition of the peroxidase and the increase in absorbance at 590 nm was monitored for 3 min. For quantitation, a standard curve was generated by using graded amount of H<sub>2</sub>O<sub>2</sub> in the reaction mixture.

#### **Antioxidant Enzymes:**

**Extraction:** The overall procedure was carried out at 0°C to 4°C according to Abdel Nasser, [29]. Samples (0.5g) of leaves tissue and roots, were ground and homogenized in 20 ml of ice-cold extraction buffer (100 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH7.8), 800 mg polyvinylpyrrolidone, 0.5% Triton X-100, 5 mM ascorbic acid). The homogenate was centrifuged at 40,000×g (20 min, 4°C). Aliquots of 3 ml of supernatant

were passed through a column filled with sephadex G-25 (PD-column-pharmacia-Germany) which had been equilibrated with elution buffer (100 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>, pH 7.0).

According to Asada [30], the elution buffer for APX contained additionally 1 mM ascorbic acid in order to keep APX enzyme in the active state. The purified extracts were used for the determination of SOD, CAT, APX and GR.

#### **Enzymes Assays:**

**SOD (EC 1.15.1.1):** Activity was measured according to the method of Misra and Fridovich [31]. One unit of SOD activity was amount of enzyme activity that inhibited epinephrine formation by 50% [31].

**APX (EC 1.11.1.11):** Activity was determined as described by Asada, [30]. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> dependent oxidation of ascorbate was followed by monitoring the decrease in absorbency with an absorption coefficient of 2.8  $\text{mM}^{-1}\text{cm}^{-1}$ . One unit of APX was expressed as micromoles ascorbate oxidized per milligram of protein per minute.

**CAT (EC 1.11.1.6):** Activity was assayed according to Aebi [32] where decomposition of H<sub>2</sub>O<sub>2</sub> is followed spectrophotometrically at 240 nm. One unit of enzyme activity is equal to 1  $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> decomposed per min.

**GR (EC 1.6.4.2):** Activity was assayed spectrophotometrically according to Smith *et al.* [33]. The reaction was initiated by the addition of GSSG. After the formation of thiobenzoic acid, the absorbance at 412 nm was measured from the linear portion of the curve, usually within 5 min. GR activity was expressed as units per milligram of protein.

#### **Non Enzymatic Antioxidant:**

**Glutathione:** GSH and total glutathione (GSH+GSSG) were assayed according to Griffith [34] and described by Abdel Nasser [29]. GSSG was determined from the difference between total glutathione (GSH+GSSG) and glutathione (GSH).

**Ascorbate and Total Ascorbate (AsA+DHAA):** Ascorbic acid (AsA) and dehydroascorbic acid (DHA) contents were determined according to the method of Law *et al.* [35]. Total ascorbate was determined through the reduction of DHAA to AsA by 0.97 mM dithiothreitol (DTT) and the DHAA concentration was determined by

estimating the difference between total ascorbate and AsA values. A standard curve covering the range 0-25  $\mu\text{mol}$  AsA was used.

**Statistical Analysis:** All estimates of sample variability are given in terms of the SE. Significant differences were determined by Student's *t*-test applied to various measured parameters of the control and plants treated with different Cd concentrations. Significance of difference was tested at  $P\#0.05$  using ANOVA. The data are means  $\pm$  SE from four determinations of at least three independent experiments

### RESULTS

**Growth Responses and Cd Accumulation:** This study showed that the application of Cd at concentrations of 10, 50 and 100  $\mu\text{M}$  caused a significant decrease ( $P\#0.05$ ) in each of the growth parameters such as fresh and dry weights of both root and shoot (Fig. 1) and leaf area (Fig. 2 A), indicating that irreversible damages to tissue formation was induced under different Cd levels and the deleterious effect of Cd became more severe with increasing Cd level and time of exposure. For example, addition of 100  $\mu\text{M}$  Cd resulted in a decrease in shoot and root dry weights by approximately 65.6 and 76.3%, respectively (Fig. 1). Leaf area was also significantly smaller than control at higher Cd concentration by 90% (Fig. 2A). In addition, the relative water content (RWC) of the leaf was decreased from 94% in control to 78, 53 and 20%, respectively at 10, 50 and 100 of Cd treatment at the end of the experiment (Fig. 2B).

Furthermore, Cd at high concentrations clearly produced a severe reduction in the shoot height and root length reaching values 85 and 82.4 %, respectively than control in response to 100  $\mu\text{M}$  Cd within 96 h of Cd exposure. On the other hand, sunflower plant exposed to 10  $\mu\text{M}$  Cd showed less pronounced responses and a small increase in the root length or shoot height (not significant). At the same time the root and shoot tissues accumulate Cd ions amounted to about 990 and 260  $\mu\text{g/g}$ . dry weight, respectively (Fig. 4). The major accumulation of Cd was found in the root particularly during the first 48 h after Cd exposure.

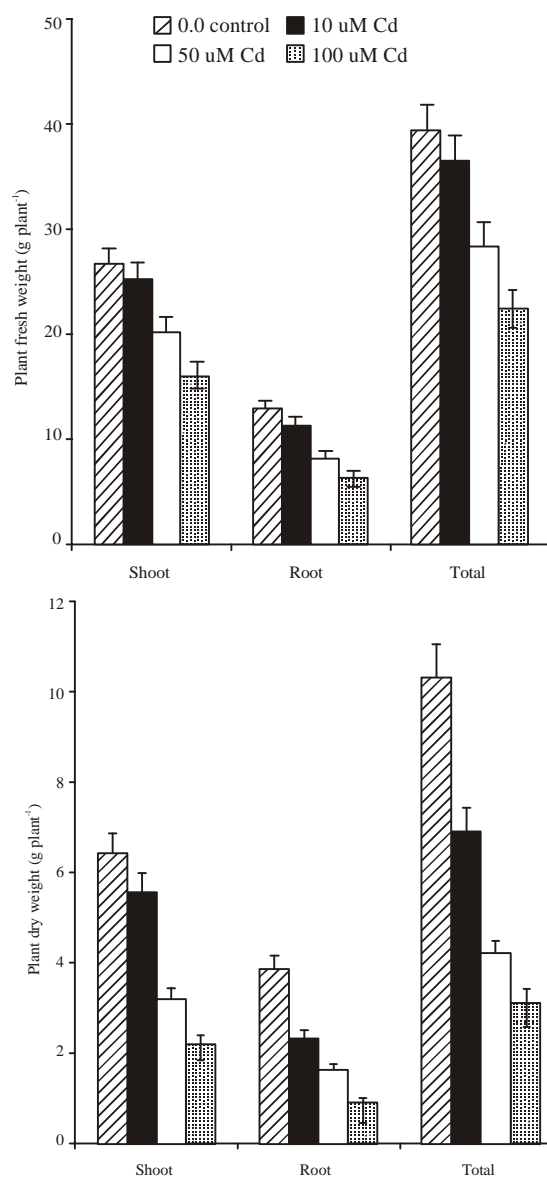


Fig. 1: Changes in fresh and dry weights of different organs of sunflower plant exposed to different concentrations of Cd for 96 hours. Values are means $\pm$ SE (n=10)

In this connection at the end of the experimental period, the leaves of Cd-treated plants showed a visual

Table1: Leaf and root symptoms of Cd toxicity of sunflower seedling under different Cd levels

Hours of Cd exposure (h)	Cd concentrations ( $\mu\text{mol/L}$ )	Leaf symptoms(chlorosis and necrotic)	Root color (Browning)
96	0	*	*
	10	*	+
	50	++	+++
	100	++++	++++

Leaf symptoms of Cd toxicity (necrotic patches on leaf blade) for Cd treatments: \* very slight or absent; + slight;; ++ mild; +++ severe; ++++ very severe

Table 2: Changes in chlorophyll contents (mg. gG'DW), chlorophyll a/b ratio and total carotenoids (mg/mg Chl) in leaves of sunflower plants treated with different concentrations of Cd. Data are means  $\pm$  SE (n=5). Values in parentheses were expressed as percentage of the respective parameters

Parameters	Cd concentrations ( $\mu$ M)			
	0	10	50	100
Chl <i>a</i>	11.4 $\pm$ 0.82 (100)	9.91 $\pm$ 0.11 (-13)	4.90 $\pm$ 0.42(-57)	2.40 $\pm$ 0.86 (-79)
Chl <i>b</i>	6.5 $\pm$ 0.31 (100)	6.20 $\pm$ 0.14 (-5)	5.75 $\pm$ 0.18 (-11)	4.20 $\pm$ 0.10 (-35)
Chl <i>a+b</i>	17.9 $\pm$ 0.66 (100)	16.11 $\pm$ 0.26(-10)	9.10 $\pm$ 0.18 (-51)	5.35 $\pm$ 0.46 (-70)
Chl <i>a/b</i> ratio	1.7	1.6	1.03	0.57
Total carotenoids	4.8 $\pm$ 0.12 (100)	15.40 $\pm$ 0.26(+13)	3.68 $\pm$ 0.12(-23)	1.93 $\pm$ 0.12 (-60)

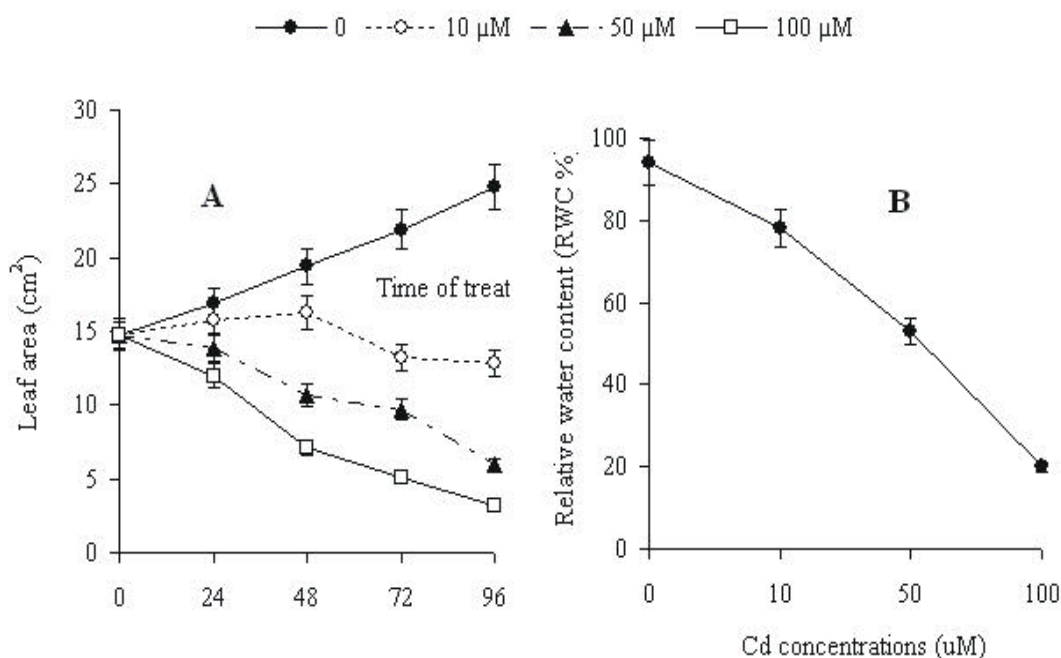


Fig. 2: Changes in the area (A) and relative water content (B) of the leaf of sunflower plant exposed to the different concentrations of Cd for 96 hours. Values are means  $\pm$  SE (n=10)

symptoms (chlorosis and necrotic) and the color of the root became brown (Table 1).

**Determination of Chlorophylls Content:** The results showed that the total chlorophyll content was reduced by about 10, 51 and 70% in leaves of plants that treated with 10, 50 and 100  $\mu$ M Cd, respectively (Table 2). This reduction was less pronounced in chlorophyll *b* than in chlorophyll *a*. In fact, the ratio of chlorophyll *a* to chlorophyll *b* tended to decrease with increasing Cd concentration. At the same time, it was found that total carotenoids content was increased at concentrations of 10  $\mu$ M, whereas at 50 and 100  $\mu$ M these contents were significantly decreased (Table 2).

**Elements Changes of the Leaves and Roots under Cd**

**Treatments:** Ionic balance inside the cell is closely related to plant adaptation to heavy metals treatments. The elements ratios under Cd treatment with different concentration. The results given in Table 3 showed that the nutrient composition of some elements in the roots and leaves of sunflower plant was altered by Cd treatments which are varied according to the concentrations of this metal. Generally speaking, it was found that Cd treatment caused a significant reduction in Fe and Mn contents. In addition, the contents of macronutrients such as Ca, Mg, N, P and K were decreased at all treated concentrations (Table 3). Cd in both leaves and roots in respect to the control.

Table 3: Changes in the content of some elements in shoot and root of sunflower plant treated with different concentrations of Cd. Data are represented as % of control (n=5)

CdConc.( $\mu\text{M}$ )	Shoot							Root						
	Elements							Elements						
	N	P	K	Ca	Mg	Mn	Fe	N	P	K	Ca	Mg	Mn	Fe
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
10	91.6	96.6	90.6	92.7	94.5	93.8	90.5	97.0	96.1	98.6	86.8	97.6	89.0	88.8
50	55.1	71.8	78.3	83.3	69.2	67.9	54.8	67.9	77.6	77.8	84.8	82.7	54.7	44.8
100	39.3	56.0	69.5	75.8	59.6	42.8	41.1	54.0	56.8	70.2	66.9	69.2	44.1	40.6

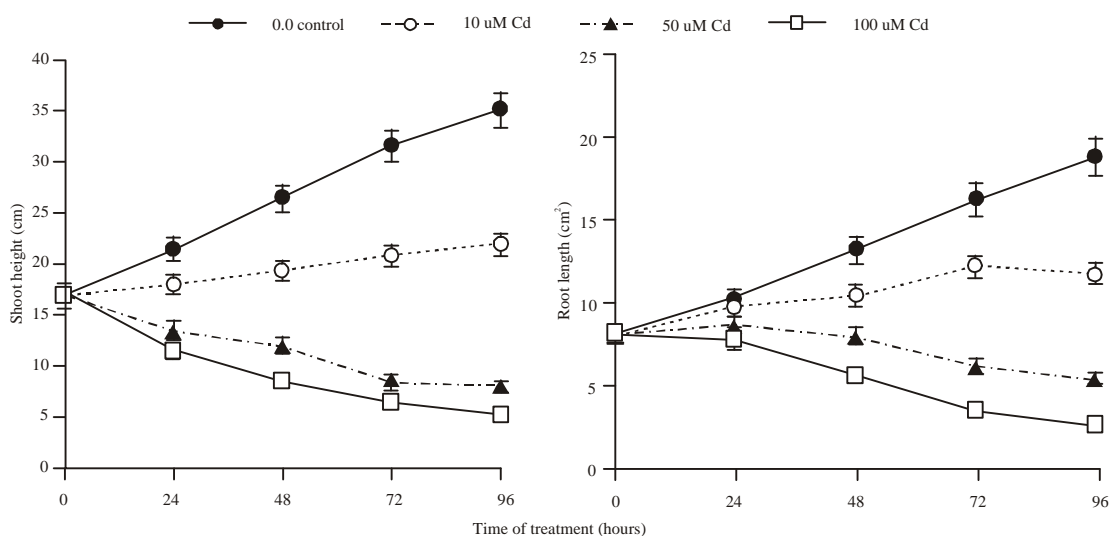


Fig. 3: Changes in the shoot height and root length of sunflower plant exposed to different concentrations of Cd for 96 hours. Values are means  $\pm$  SE (n=10)

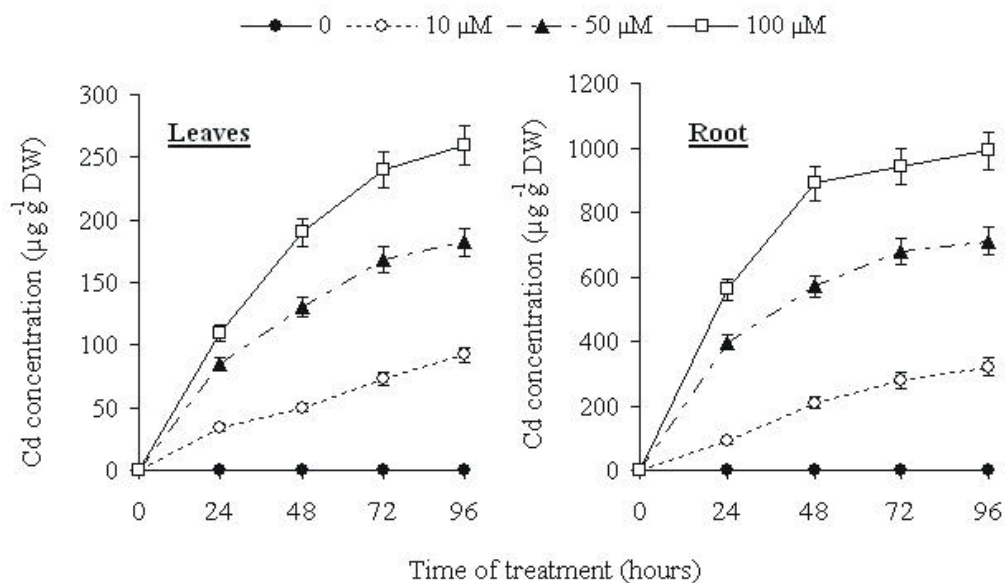


Fig. 4: Cd concentration in leaves (A) and roots (B) of sunflower plant exposed to different concentrations of Cd for 96 hours. Values are means  $\pm$  SE (n=10)

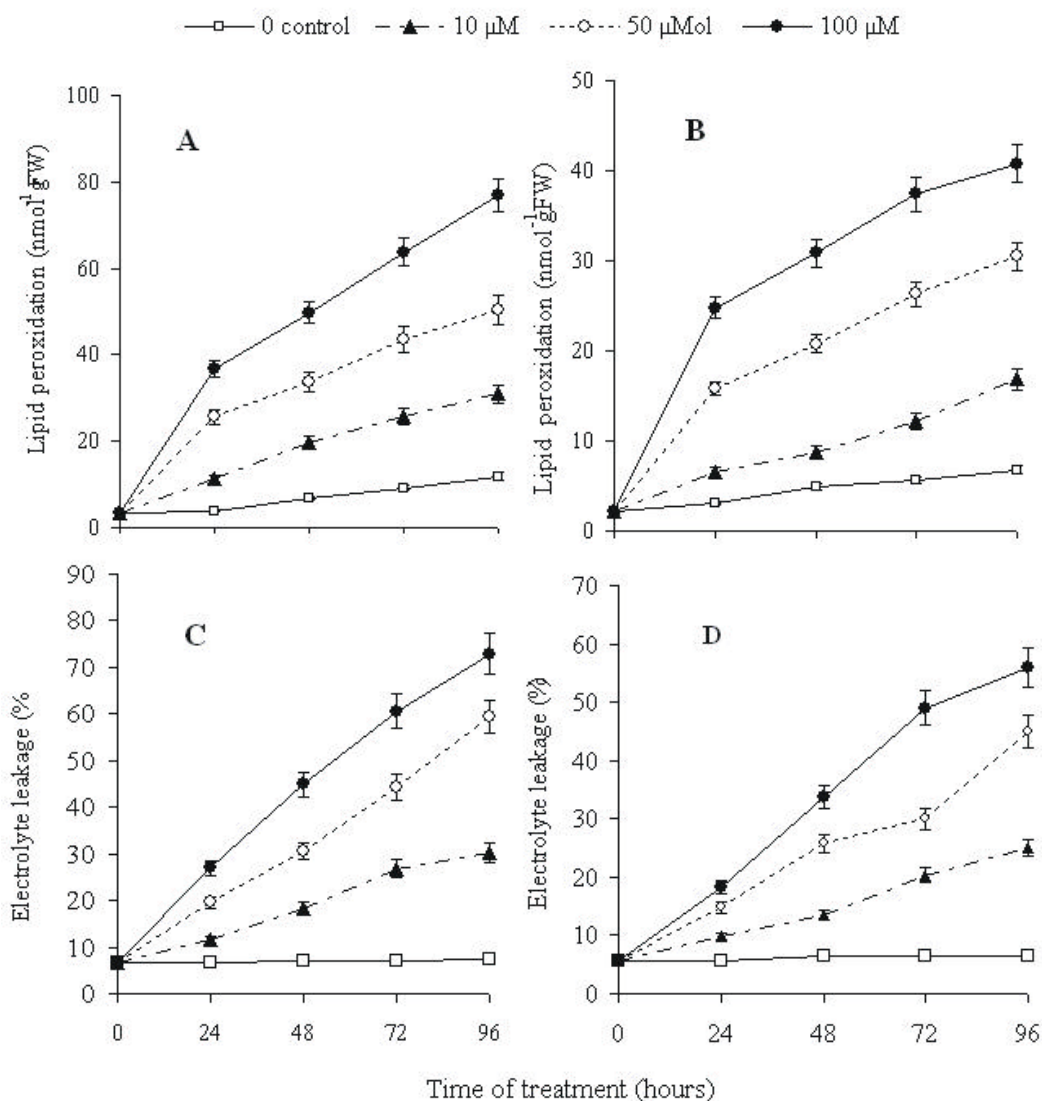


Fig. 5: Effect of different concentrations of Cd on lipid peroxidation (MDA production) in leaves (A) and in roots (B); and electrolyte leakage in leaves (C) and roots (D). Values are means  $\pm$  SE (n=5)

**Lipid Peroxidation and Membrane Permeability in Response to Cd Treatments:** Malondialdehyde (MDA) level was used as indicator for lipid peroxidation and represents a balance of oxidative stress that induced production of MDA in relation to Cd treatments. Thus, MDA can be regarded as a sink for oxidative radical. However, the results given in Fig. 5 A and B showed a marked increase in MDA level as a result of Cd treatment and the increase in Cd concentration enhanced the degree of lipid peroxidation. For convenience, during the exposure time, control seedling (leaves and roots) only 11.9 and 6.8  $\mu\text{mol}$  MDA / g. fresh weight, respectively.

Because of Cd induction to a generation of oxidative stress, this study showed that membrane permeability (MP) of leaves and roots tissues caused membrane injury and electrolyte leakage as a result of oxidative damage induced by Cd treatments. As shown in Fig. 5 C and D. Electrolyte leakage was increased by 11 and 10 fold in the leaves and roots respectively treated with 100  $\mu\text{M}$  Cd, compared to control, the corresponding figures at 50  $\mu\text{M}$  were 9 and 8 fold, respectively.

**Hydrogen Peroxide Content as Affected to Cd Treatments:** The amount of hydrogen peroxide  $\text{H}_2\text{O}_2$  (a product of superoxide dismutase reaction) showed

Table 4: Effect of different concentrations of Cd on endogenous levels of reduced ascorbate (DH.AA), oxidized ascorbate (AsA), reduced glutathione (GSH) and oxidized glutathione (GSSG) in sunflower leaves

Cd Conc (μM)	AsA	DHA μmol g <sup>-1</sup> FW	AsA+DHA	AsA/DHA	GSH μmol g <sup>-1</sup> FW	GSSG	GSH+GSSG	GSH/GSSG
0	50.8±3.4 a	100.8±6.2a	151.6	0.50	58.3±2.4 a	34.8 ± 1.2 a	93.1	1.67
10	40.6±2.4b	126.5±4.2b	166.6	0.32	50.4 ± 3.4 b	33.0 ± 1.2b	92.9	1.52
50	20.1±3.2 c	164.8±5.6 c	184.8	0.12	60.8 ± 6.9 c	28.8 ± 3.6 c	85.6	2.11
100	10.2±0.4d	180.5±6.2 d	190.5	0.06	64.0 ±2.9c	15.4 ± 4.2d	84.4	4.15

Values are means ±SE (n=5). Means within the column with the same letter are not significantly different ( $P > 0.05$ ). \* is significant at  $P < 0.01$

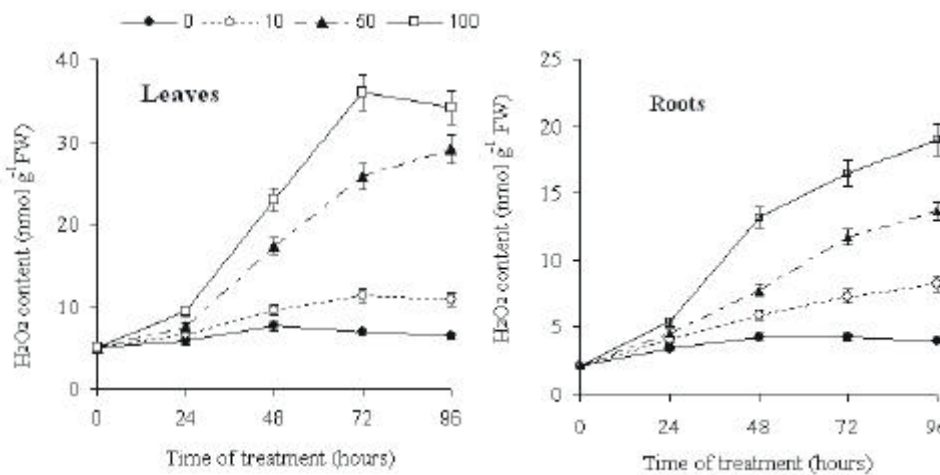


Fig. 6: Effect of different concentrations of Cd on H<sub>2</sub>O<sub>2</sub> content in the leaves of sunflower plant treated with Cd for 96 h. Values are means ± SE (n=5)

a significant increase in leaves and roots of sunflower plant treated with Cd particularly at higher concentrations compared to untreated plants and was more pronounced in leaves than in roots (Fig. 6). The level of H<sub>2</sub>O<sub>2</sub> in roots of Cd-treated plants gradually increased from 6% (at 10 μM) and 49% (at 100 μM) after 48 h to 10.5% (at 10 μM) and 62% (at 100 μM) over a period of 96 hours. Similar trend also occurred in leaves with a significantly higher increase that obtained in roots that ranged from 10-17% at 10 μM Cd and 56-77% at 100 μM Cd.

#### Responses of Antioxidant Systems to Cd Treatment:

To study the stress response in plants exposed to different cadmium concentrations, the changes in activities of some enzymatic and non-enzymatic antioxidant were analyzed in the leaves and roots (Figs. 7A, B, C and D).

#### Enzymatic and Non-enzymatic Antioxidant Activities in Response to Cd Treatments:

**Antioxidant Enzymes:** Activities of antioxidant enzymes were determined over an experimental period of 96 h in Cd-treated leaves. In this period, enzymes activities of controls were more or less around their means. However, SOD enzyme activity in leaves treated with 50 or 100 μM

Cd was stimulated during the first 48 h, then decreased significantly at the end of the experiment (Fig.7 A), whereas, exposure to 10 μM Cd had no significant effects on SOD activity.

On the other hand, during the first 24 h, CAT and APX activities were initially significantly suppressed by Cd treatments (Fig. 7B and C), but after 48 h, these activities were strongly increased particularly at 50 and 100 μM Cd. At the end of the experiment, the activities of the CAT and APX enzymes were declined. Concerning the activity of GR it was found that cadmium treatment at the concentrations of 10, 50 and 100 μM caused a significant inhibition of GR activity within the first 24 h, whereas this activity was approximately 3 fold increases than the controls at the end of experiment (Fig. 7D).

#### Non Enzymatic Antioxidants (Ascorbate and Glutathione Contents) in Response to Cd Treatments:

The results presented in Table 4 showed that total ascorbate (ascorbate; AsA + dehydroascorbate DHA), was significantly increased in leaves of sunflower plant exposed to Cd at 10, 50 and 100 μM than the controls. At the same time, the level of AsA was markedly decreased to very low level particularly at high concentration (100 μM Cd) in the leaves, with a

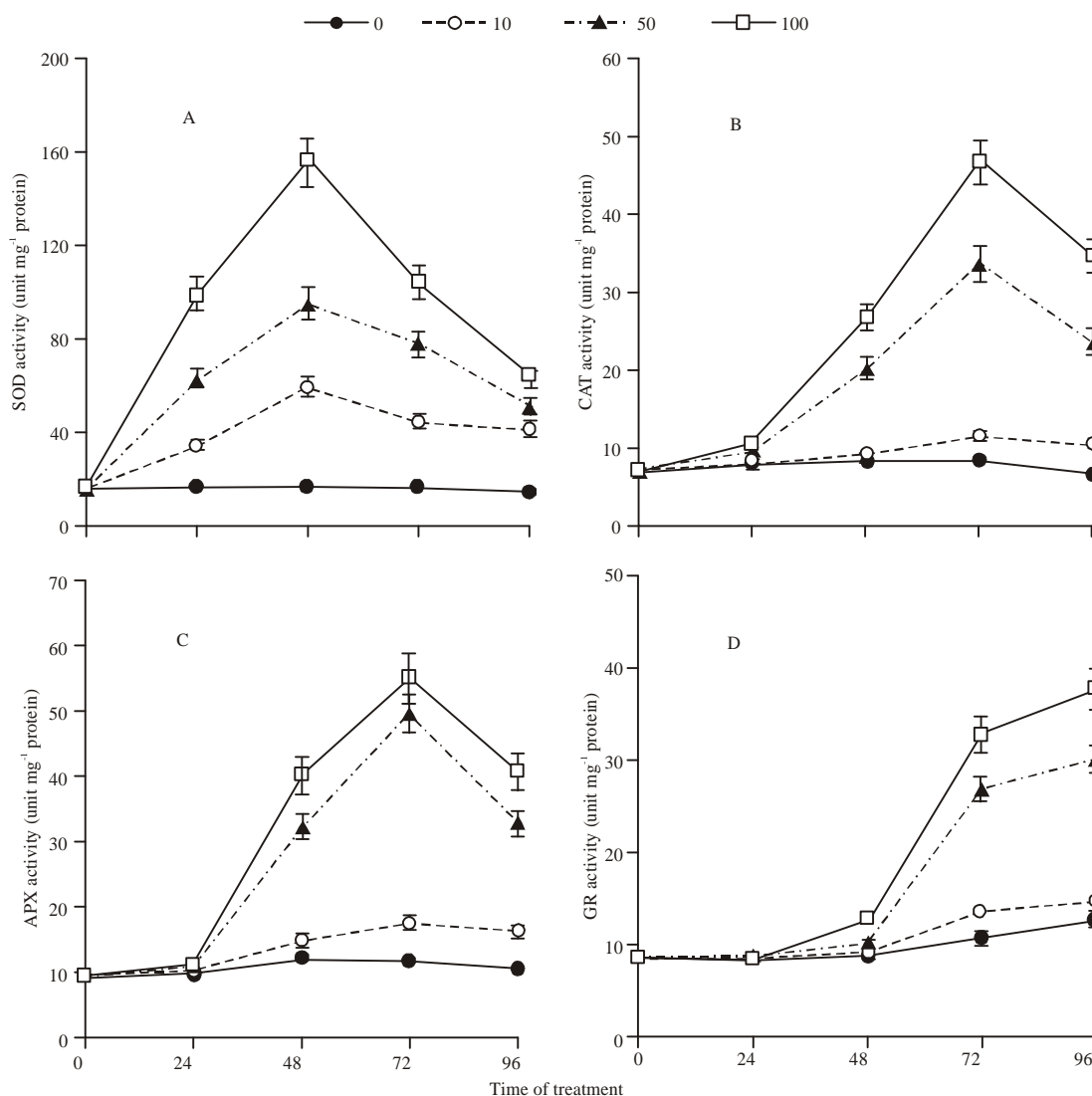


Fig. 7: Effect of different concentrations on the activities of antioxidant enzymes SOD (A), CAT (B), APX (C) and GR (D) in leaves of sunflower plant treated with Cd for 96 h. Values are means + SE (n=5)

corresponding increase in the level of oxidized form (DHA) amounted to approximately from 26 to 80% at 10 and 100 μM of Cd compared to control (Table 4) indicating that DHA content was significantly enhanced.

Furthermore, the accumulation of glutathione (GSH) in the leaves was observed as response to Cd effect; a maximum level was reached at 100 μmol Cd treatments. Whereas, the result shows that glutathione disulphide (GSSG) was significantly decreased. Accordingly, the GSH/GSSG ratio was increased, indicating that glutathione pool appeared to be more reduced after cadmium exposure for 96 h as compared to the control plant.

## DISCUSSION

Several studies demonstrated that cadmium ions can function as stressor, causing some physiological constraints that decrease plant vigor and inhibit plant growth [36, 37]. Also, phototoxic symptom such as root browning [38] was observed (Table 1).

The results showed that reduction in the fresh and dry weights of both root and shoot, leaf area and leaf water content were among the most sensitive responses to Cd exposure and faster than most of the other physiological reactions analyzed (Fig. 1 and 2). Also, the results presented in Fig. 3 showed an inhibition in the root length by 37.2, 50 and 86 % of control at 10, 50 and 100 μM Cd respectively.

100  $\mu\text{M}$ . In the shoot the corresponding values were 39, 78 and 85.5%, respectively (Fig. 3).

In this connection, Ouzounidou *et al.* [39] suggested that the inhibitory action of heavy metals on root length, shoot height and leaf area seems principally to be due to chromosomal aberrations and abnormal cell divisions and may also be correlated with the metal-induced inhibition of photosynthetic process and the respiration in the shoot system and protein synthesis in the root, or due to the reduction in cell proliferation and growth [17, 40].

Cadmium as toxic metal is one of the most readily absorbed and accumulated in the plant (Fig. 4) was occurred within 96 h at Cd concentrations in the root in the range between 220 and 990  $\mu\text{g Cd/g}$  dry weight as judged from the 10 and 100 M Cd treatments, regardless of the concentration applied. Whereas, the corresponding figures in the leaves were 92 and 260  $\mu\text{g Cd}$  (Fig. 4). This observation suggests that Cd uptake by root is counter balanced by transport to leaves indicating that root growth was inhibited very early. Similar results were obtained by Schützendübel *et al.* [37] on *Pinus sylvestris* and poplar seedling roots supplied with 50  $\mu\text{M Cd}$  [16].

Differences in the degree of expressed phytotoxicity were established due to the various Cd-concentrations applied to the plant nutrient medium and the duration of the treatment. The symptoms of phytotoxicity were expressed more clearly in the roots and leaves (Table 1) because of the significantly higher heavy metal accumulation [41, 42].

Moya *et al.* [43] reported that the negative effect of Cd on plant growth was accompanied by an increase in dry to fresh mass (DM/FM) ratio in all organs. Despite that DM/FM ratio was changed during ontogenesis, its sharp increase in young plants is a criterion for stress response, which is indicative on whole plant level [44].

**Chlorophyll Contents:** A typical sign of Cd toxicity is the decrease of chlorophylls contents as observed in this study. It was established that Cd applied *in vivo* decreased the pigments content [45]. It was found that concentration of Chl *a* was reduced more than that of Chl *b* and carotenoids (Table 2). However, the visible symptoms of injury observed in this investigation such as chlorosis and necrosis (Table 1) can be related to the chlorophyll loss. Most researchers connected the reduction of chlorophyll in Cd-treated plants to the inhibition of its biosynthesis [40, 46, 47].

In this connection, Fabrizio *et al.* [46] reported that a typical sign of Cd toxicity is visible loss of chlorophylls

in leaves of *Phragmites australis* and that vegetables growing in medium with high level of Cd showed deleterious effect in photosynthetic processes, such as decrease of chlorophyll content and photosynthesis. Lately inhibiting effect of Cd was established in other species such as wheat [47] and bean [48].

However, the results of this study could be interpreted as the effect of strong oxidation due to the presence of Cd on the photochemical apparatus [49], reduction of chloroplast density and size [17] or probably due to the effect of interference of Cd with Fe root uptake [50], and also that Cd could have replaced Mg in chlorophylls [51].

**Lipid Peroxidation:** Lipid peroxidation was determined as malondialdehyde (MDA) level. The cellular level of MDA represents a balance of oxidative stress induced production of MDA. Thus, MDA can be regarded as a sink for oxidative radical. The results given in Fig. (5 A and B) showed that Cd treatment induced a marked increase in MDA level as a result of Cd treatment indicating that this element like other environmental stresses can generate the production of a powerful oxidation which in turn brings lipid peroxidation.

In this connection, Savoure *et al.* [52] reported that lipids are very sensitive to hydroxyl radicals ( $\text{OH}^\cdot$ ) and the presence of lipid peroxidation is one of the first signs of oxidative stress. Direct evidence from our study suggested that Cd generates free radicals, which increase the lipid peroxidation and disruption of membrane integrity and lead to increase of ions leakage (Fig. 5C, D). Similar results were obtained by Sanita di Topi and Gabbrielli [53]. Ouzounidou *et al.* [36] assumed that heavy metals induces indirectly depolarization of the membrane potential as a result of depletion of electrogenic  $\text{H}^+$ -pump caused by proton uptake into the inner cellular space.

Membrane damage was evaluated indirectly with electric conductivity (EC) measurements of solute leakage from the cells [54]. Interestingly, in the present study the leakage of ions was only increased upon addition of  $\text{Cd}^{2+}$  particularly at the highest concentrations demonstrating that the structure and functions of plasma membrane were rapidly affected by this metal. For example, it was shown that Cd treatment caused a decrease in the efflux of K, Mn, N, P, Ca, Mg and Fe from roots and leaves of sunflower plant. However, such damage could result from various mechanisms including the oxidation reactive by oxygen species or the changes in cell permeability which may attributed to non-selective conductance increases and inhibition of the  $\text{H}^+$ -ATPase

activity of the plasma membrane fraction of wheat and sunflower roots [55] and [56] to probably due to the changes in the composition and fluidity of membrane lipids [57]. In this connection, it has been reported by Mishra *et al.* [58] that Cadmium, like other classes of metals, has strong affinity towards nitrogen-and sulfur-containing ligands and proteins. So, it forms bridges within proteins leading to distorted membrane ion channels and leakage of ions.

Similarly, increased leakage of ions from roots or leaves has been reported by other investigations working with other plant species exposed to a range of Cd concentrations [54, 58, 59]. Furthermore, others investigators concluded that damage to the plasma membrane, monitored by ion leakage as a symptom of injury, was the primary cause of Cu toxicity in the cells of the roots of *Silene vulga* [60] and wheat [61]. In addition, it has been reported that many plant species grown in high Al level usually decreased significantly the concentrations of N, P, Ca, Mg and Fe [62, 63]. In spinach plants, Ouzounidou *et al.* [36] reported that Fe, Na, K, Ca and Mg content declined under Cu treatment.

**Hydrogen Peroxide:** H<sub>2</sub>O<sub>2</sub> play roles as signals for the induction and regulation of stress enzymes such as APX [64, 65]. However, in this study it was found that Cd at high concentration resulted in a sever oxidative stress because the H<sub>2</sub>O<sub>2</sub> was greatly accumulated (Fig. 6). However, this is theoretically possible when the activities of APX or CAT are decreased [66, 67]. Doke *et al.* [68] has reported that accumulation of H<sub>2</sub>O<sub>2</sub> is a general stress response, which has been observed in plants exposed to Cd ions, low temperature, heat, pathogens and chilling.

**Responses of Antioxidant Systems to Cd Treatment:** Plant cells posses several enzymatic and non-enzymatic antioxidative systems preventing production of OH<sup>•</sup> radicals [69]. The time courses of antioxidative responses after Cd exposure suggest that the following sequence of events may take place: Initially, Cd uptake leads to a depletion of glutathione and inhibits CAT, APX and GR [67].

**Antioxidant Enzymes:** The activity of antioxidant enzymes such as superoxide dismutase (SOD) which controls the concentration of O<sub>2</sub> and its derivatives (OH<sup>•</sup> and H<sub>2</sub>O<sub>2</sub>) is defined as a key antioxidant enzyme of plants [69, 70]. In addition, catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) have

been studied in the leaves of sunflower plant in response to Cd treatment (Fig. 7 A, B, C and D). In this study it was found that Cd concentrations at 50 and 100µM initially increased SOD during the first 48 h and then decreased significantly at the end of the experiment, whereas, exposure to 10 µM Cd had no significant effects on SOD activity (Fig. 7A). However, the behavior of SOD did not show the same pattern of variation in all heavy metals treated plants due to the presence of different isozymes due to some adaptive changes in the activities responsible for scavenging of active oxygen species to mitigate the situation, Alscher *et al.*[71].

Figures 7B and C showed that Cd metal may inhibit other enzymes involved in H<sub>2</sub>O<sub>2</sub> removal such as CAT and APX and consequently caused H<sub>2</sub>O<sub>2</sub> accumulation. CAT and APX activities were initially significantly suppressed by Cd, but after 48 h, these activities were strongly increased at 50 and 100 µM Cd. At the end of the experiment, the activities of the CAT and APX enzymes were declined. This could be due to the binds of Cd metals with thiol groups and thereby inactivates the activities of these enzymes [72]. The same mechanism of inhibition was obtained with thiol containing enzymes such as GR [68]. In this connection, Romerio-Puertas *et al.* [70] suggested that Cd simultaneously inhibited the systems involved in H<sub>2</sub>O<sub>2</sub> removal such as CAT, APX and GR and resulted in elevated activities of SOD and such a situation must inevitably lead to the accumulation of H<sub>2</sub>O<sub>2</sub>.

Figures 7B and C showed that after 48 h, the activities of CAT and APX enzymes were stimulated up to 72 h, then declined. Mullineaux and Creissen [73] reported that there is a reduction in CAT activity in the leaf of cucumber seedling upon exposure to heavy metals. Similar pattern was observed with glutathione reductase (GR) activity during the first 24 h increased until the end of experimental period (Fig. 7). Cuyers *et al.* [74] found that the increase was related to increased synthesis of GR protein because the transcript levels were increased after Cd exposure.

**Ascorbate and Glutathione Contents:** The ascorbate-glutathione cycle seems to be a mechanism of great importance in controlling the cellular redox status, especially after application of heavy metals such as copper, zinc and cadmium [74-76]. Both ascorbic acid (AsA) and glutathione (GSH) in the oxidized and reduced forms are among the most important non-enzymatic cellular antioxidant defense compounds by quenching ROS and are important in controlling the metal

homeostasis [71]. The ratio of ascorbic acid in the reduced form (AsA) to that in the oxidized form (DHA) is considered an important indicator of the redox state of the cell and the degree of oxidative stress experienced.

Table 4 showed that total ascorbate (AsA+DHA), was significantly increased at 10, 50 and 100  $\mu\text{M}$  Cd compared with controls. At the same time the level of AsA was markedly decreased to a very low level at high concentration (100  $\mu\text{M}$  Cd). With a corresponding increase in the level of reduced form (DHA) indicating that DHA content was significantly enhanced probably through glutathione-dependent reaction by DHAR enzyme and/or due to the decrease in AsA synthesis [29]. The strong increase in DHA level can be explained by its antioxidative role in quenching peroxides such as  $\text{H}_2\text{O}_2$  to function as an antioxidant,

The conversion of GSSG to GSH which catalyzed by GR enzyme was correlated with the change in GSH/GSSG mole ratios which plays an important role in cellular redox status and in signal transduction of several transcription and metabolic processes. The accumulation of glutathione (GSH) in the leaves was observed as response to Cd effect, with a maximum level at 100 Cd treatments (Table 4). Also, the results show that glutathione disulphide (GSSG) was significantly decreased in the leaves. Accordingly, the GSH/ GSSG ratio was increased, indicating that glutathione (GSH) pool appeared to be more reduced after cadmium exposure for 96 h as compared to the control plants.

According to Asada *et al.* [76] GSH plays a key role in limiting cellular damage and induction of defense mechanisms against activated species of oxygen in response to the heavy metals treatments. This confirms the suggestion that GSH can trap Cd only when they are in the reduced state. Smeets *et al.* [75] reported that the affinity of heavy metals such as Cd to bind to GSH, forming metal-thiolate compounds, suggesting that GSH might be involved in the synthesis of phytochelating which could detoxify Cd ions. Therefore, the high GSH/GSSG ratio in response to Cd seems to be necessary not only for the role of glutathione as reductant (GSH), but also to achieve optimal protein synthesis in the plant cells. In this study, GSH/GSSG ratio was increased as the concentration of Cd increased from 10 to 100  $\mu\text{M}$  due to the strong decrease of GSSG and to the high activity of GR enzyme (Table 4). In contrast, Cuypers *et al.* [77] reported that oxidation of the GSH pool to GSSG will cause a signal transduction failure and homeostasis will be disturbed suggesting that Cd can be added to the list of stresses such as drought, heat shock, ozone and  $\text{SO}_2$ .

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