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Calcium maintains membrane integrity but neither glutathione nor ascorbate redox poise of *Vicia faba* L. radicle cells exposed to sub-lethal cadmium dose onset of germination

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Biochemical reactions to Cd, in absence or presence of exogenously applied Ca, in the radicle and cotyledons of *Vicia faba* L. seeds were studied to gain insight into the early events after Cd exposure in order to characterize the physiological background of the metal response. Membrane integrity, changes in; redox state, activities of the enzymes responsible for retrieving the reduced forms and for detoxification of H_2O_2 were estimated. Sub-lethal Cd dose resulted in GSH reduction, in radicle tissues, by 51.0 %, since GR activity was significantly decreased. The redox state for both antioxidants as well as GSH/GSSG and Asc/DHA ratios were significantly diminished. Therefore, the redox poise of the radicle cells directed towards sever oxidative load. Cd exposure resulted in a significant inhibitory effect on activities of antioxidative enzymes; SOD; CAT; GR, but had non-significant effect on APx and DHAR activities. Calcium alleviates the oxidative stress on membranes, represented in lowered lipid peroxidation and K leakiness. In cotyledons, Ca increased Asc and GSH pools over the control. Exposure to Ca in addition to Cd significantly increased SOD and DHAR activities. The presented data indicate that Cd acted via the disturbance of the cellular redox control and Ca may share in apoptotic signaling pathway. It can be concluded that Cd was able to break down the protective barrier of the Asc-GSH cycle and its complements. The changes in the enzyme activities could be related to the Cd-induced promotion of oxidative signals that favored programmed cell death as evident from mitotic investigation.

Keywords: Ascorbate–glutathione cycle, Cd and Ca, faba bean, membrane damage, oxidative stress, redox poise.

INTRODUCTION

Cadmium is a toxic heavy metal pollutant with long biological half-life. It has no biological function, but it is readily taken up by the roots of many plants because of its high mobility and solubility (Wagner, 1993). According to the chemical and physical properties of heavy metal ions, their harmful action can be divided into: a) substitution of essential metal ions by other incorrect ones, b) blocking of essential functional groups in biomolecules; proteins and polynucleotides (Schützendübel and Polle, 2002)and c) generation of reactive oxygen species (ROS) by auto-oxidation and Fenton and Haber-Weiss reactions (Repetto et al., 2010).Although Cd is not able to trigger Fenton-type reactions, it can indirectly activate the plasma membrane NADPH oxidase, thus inducing generation of ROS in the exposed tissues(Bielen et al.,2013).Olmos et al., (2003)demonstrated a rapid generation of hydrogen peroxide(H_2O_2)byCd^{2+}-treated tobacco cultured cells (BY-2 cells). By using cytochemical methods, they had located the starting point for the generation of H_2O_2 at the cell plasma membrane. Exogenous application of imidazol, an inhibitor of the neutrophil NADPH oxidase, prohibited the

production and propagation of H_2O_2induced by Cd^{2+}. The exposure of plants to toxic levels of heavy metals triggers a wide range of physiological and metabolic changes. Formation of ROS; superoxide ($O_2\cdot^-$), hydroxyl radicals (·OH) and H_2O_2in plants due to biotic and abiotic stresses was early confirmed. Lipid peroxidation is a complex process known to occur in both plants and animals, which is indicative on oxidative stress. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products. Malondialdehyde (MDA) is a major final product of lipid peroxidation (Dianzani and Barrera, 2008).Cadmium has been shown to damage cell membranes and thus it enhances the ionic leakage of plant cells(Milone et al., 2003).

Even under optimal conditions, many metabolic processes, including the chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems of higher plants, produce ROS. Furthermore, the excess concentrations of ROS can congregate as a consequence of biotic and abiotic stress conditions, resulting in oxidative damage at the cellular level or oxidative signaling to activate defence mechanisms. Whether ROS will act as damaging or signaling factors depends on the balance between their production and scavenging. Therefore, antioxidants and antioxidative enzymes function to interrupt the cascades of uncontrolled oxidation(Bielen et al., 2013). Glutathione (GSH) is the most abundant non-protein thiol in plant cells, containing a free thiol group on cysteine. Cadmium has a high affinity for the thiol group of GSH. Thiols are able to undergo redox reactions, providing GSH with both chelating and antioxidative properties (Jozefczak et al., 2015).In plants, antioxidative responses are largely based on the use and recycling of both GSH and ascorbate (Asc), complemented by superoxide dismutase (SOD) and catalase (CAT). The SODs constitute the first line of defence against $O_2\cdot^-$. These isoenzymes are classified into three groups based on their metal co-factor: iron, copper/zinc and manganese SODs. These redox-active metals dismutate $O_2\cdot^-$ and produce the relatively stable H_2O_2. Both CAT and ascorbate peroxidase (APx) are major H_2O_2 scavengers preventing the formation of toxic ·OH (Halliwell, 2006). CAT has a low affinity for H_2O_2 but a high reaction speed. These different characteristics make APx is the best for ROS fine tuning, it exists as isozymes and plays an important role in the metabolism of H_2O_2.CAT might be responsible for removing the excess of ROS (Cuypers et al., 2011). In the Asc–GSH cycle, both metabolites are successively oxidized and reduced allowing APx to reduce H_2O_2. Oxidized forms of Asc [dehydroascorbate (DHA)] and GSH [glutathione disulphide (GSSG)] are recycled by dehydroascorbate reductase (DHAR) and glutathione reductase (GR) using GSH and NADPH as electron donors, respectively (Jozefczak et al., 2012). Ascorbate also directly neutralizes ROS non-enzymatically. In order to maintain protein structure and function, a reducing intracellular milieu is essential. Both GSH/GSSG and Asc/DHA pairs are major cellular redox buffers. Alteration in the ratios of the reduced and oxidized forms reflect cellular toxicity and have been associated with redox signaling (Zagorchev et al., 2013).

Calcium is an essential nutrient that participates in many biological response systems of plants. It counters Cd uptake and alleviates metal toxicity stress (Ismail, 2008). Plasma membrane is the first target for heavy metal toxicity in plants and its destruction alters normal Ca signal transferring system. Therefore, application of moderate concentrations of exogenous Ca can protect plants from environmental stresses by enhancing plasma membrane stability and restoring Ca signal transferring system (Wan et al., 2011). There have been reports about the role of exogenous application of Ca for alleviating Cd toxicity in beans(Ismail, 2008) and citrus (López-Climent et al., 2014).

In the present study, both the concentration and redox state of Asc and GSH, as well as enzymatic activities of the pathway for recycling their oxidized forms and oxidative stress markers were investigated in cotyledons and radicles of 72 h Cd-exposed bean (*Vicia faba* L. cv. Giza 843) seeds onset of germination. Ca signaling was also evaluated to make a panorama for early abiotic stress (Cd in absence or presence of exogenously applied Ca) induced responses. The antagonistic role of Ca in alleviating Cd toxicity during early germination phase has been focused.

MATERIALS AND METHODS

Plant material and experimental design:

Seeds of bean (*Vicia faba* L. cv. Giza 843) were obtained from the Breeding Program of the Agricultural Research Center, Giza, Egypt. Seeds

were sterilized with 2% sodium hypochlorite for 10 minutes then rinsed thoroughly and soaked in aerated distilled water for 12 h at room temperature. Seeds were germinated in large Petri dishes (14 cm in diameter) at 27°C ±2 in the dark for 72 h over three sheets of filter papers moistened with distilled water (control) or aqueous solution of 250 µM Cd as $Cd(NO_3)_2$ alone or in combination with 500 µM Ca as $CaCl_2$. Dishes were distributed in complete randomized block design. Germinated seeds were recorded until the maximum germination of the control was obtained. Germination index (IG) was calculated as a germination percentage x L/L_0 (L, radicle length of treated seeds; L_0,radicle length of control seeds). At harvest, the seed coat was removed and the radicle (R) samples were taken 1-1.5 cm from the tip and cotyledon (C) samples were taken with a cork borer at the nearest part to the cotyledonary stalks and stored at -80°C until analyses. Samples for metal ion (Cd^{2+} and Ca^{2+}) determination were washed for 10 minutes with 10 Mm ethylene-diamine-tetraacetic acid (EDTA) for exchange of apoplastic Cd^{2+} and Ca^{2+} then with deionized water and dried at 70°C until constant weight and sampled for the analysis. The experiment was repeated several times for various analyses. Root apices (1–2 mm) were cut after 72 h of Cd (without or with Ca) exposure, onset of germination, in addition to the control for mitotic index (IM) determination. The IM was investigated according to Sharna and Sharna (1980) and expressed as the percentage of dividing cells.

Stress Markers:

Lipid peroxidation:

Malondialdehyde (MDA);the final product of lipid peroxidation in radicle tissues was determined as 2-thiobarbituric acid (TBA) reactive substances (TBARS) as described by Li (2000). Calculation of MDA equivalent was based on this formula: [C (µ mol L^{-1}) = 6.45(A532 – A600) – 0.56 (A450)]. Lipid peroxidation was expressed as concentration of MDA equivalent (nmol g^{-1} fresh weight).

Leakage of K ions:

The leakage of potassium ions was performed according to Milone et al. (2003) with slight modifications. Potassium released was measured by flame photometer, PFP 7, Jenway, UK. K^+ leakage was calculated as % of total leaked K^+.

Extraction and assay of NADHoxidase:

The enzyme (NADPH oxidase; EC 1.6.3.3) was extracted as described by Askerlund et al.(1987). NADPH oxidase activity was assayed by oxidation of NADPH at 340 nm and calculated using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as µM min^{-1} mg^{-1} protein.

Extraction and assay of reduced and oxidized ascorbate and glutathione:

For ascorbate and glutathione determination, 0.2 g of fresh tissues (radicle and cotyledons) were homogenized with cold 5% meta-phosphoric acid at 4°C at a 1 : 6 ratio (w/v) in order to obtain deproteinized extracts. After centrifugation at 15,000 x g for 15 min at 4 °C, the supernatants were collected and used for the analysis of ascorbate and glutathione concentration.

Total Glutathione Assay Kit (NWLSS™ NWK-GSH01 PRODUCT INSERT)is a quantitative assay for measuring the total glutathione pool within a sample (GSH + GSSG), which matches with that adopted by Anderson (1996) with some modifications. Glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. Concentration of GSH was determined colorimetrically with 5.5'-dithiobis-2-nitrobenzoic acid(Boyne and Ellman, 1972). Concentration of oxidized glutathione (GSSG) was calculated as the difference between total glutathione pool and GSH concentration. The results were expressed as nmol g^{-1} fresh weight. Ascorbate (Asc) and dehydroascorbate (DHA) were estimated as shown by Kampfenkel et al.,(1995) with minor modifications. Briefly, total ascorbate was determined after reduction of DHA to Asc with dithiothreitol (DTT), and concentration of DHA was estimated from the difference between total ascorbate pool (Asc plus DHA) and Asc. A standard curve was developed based on Asc in the range of 0-100 nmol /ml. The results were expressed as nmol g^{-1} fresh weight.

Extraction and assay of antioxidative enzymes:

Enzyme extract was prepared by homogenizing 0.3 g of frozen (radicle and cotyledons) tissues in 10 ml of sodium phosphate buffer (0.1 M, pH 7.0). The homogenate was centrifuged at 15,000 g at 4°C. The supernatant was collected, stored at -80°C and used as the

enzyme extract for analyses of superoxide dismutase, catalase, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase.

In order to determine the SOD (EC 1.15.1.1) activity, an indirect method using nitro-blue tetrazolium (NBT) is commonly used (Beauchamp and Fridovich, 1971) due to its ease of use. There are several disadvantages to the NBT method. SOD Assay Kit-WST (Cat.no 19160 SOD Sigma-Aldrich) allows very convenient SOD assaying by utilizing a highly water-soluble tetrazolium (WST) salt that produces a water-soluble formazan dye upon reduction with a superoxide anion ($O_2^{\cdot-}$). The activity was expressed as enzyme units mg^{-1} protein. One unit of the enzyme activity was defined as the amount of enzyme required for 50% inhibition of the reduction of WST salt in comparison with the well lacking the enzyme.

Catalase (CAT) (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity was measured as the method of Cakmak and Marschner (1992). The activity was determined by measuring the rate of disappearance of H_2O_2 for 1 minute at 240 nm, and calculated using an extinction coefficient; ϵ= 39.4 $mM^{-1}cm^{-1}$ and expressed as enzyme units mg^{-1} protein. One enzyme unit was defined as the amount of enzyme required to oxidize 1 µM of H_2O_2 min^{-1} mg^{-1} protein.

Estimation of enzyme activities of ascorbate-glutathione cycle:

Ascorbate peroxidase (APX) (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) activity was assayed as in Nakano and Asada (1981) method. The enzyme activity was determined (ϵ = 2.8 $mM^{-1}cm^{-1}$) by measuring the decrease in absorbance at 290nm for 1 minute. Dehydroascorbate reductase (DHAR) (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) activity was assayed as adopted by Hossain and Asada (1984) with slight modifications. The reaction was initiated by the addition of the enzyme extract to the assay mixture at room temperature and then absorbance was monitored at 265 nm (ϵ = 14.0 mM^{-1} cm^{-1}). Glutathione reductase (GR) (NADPH: glutathione disulphide oxidoreductase, EC 1.6.4.2) activity was determined by monitoring GSSG dependent oxidation of NADPH according to Foyer and Halliwell (1976). Absorbance at 340 nm was read immediately after addition of the enzyme extract at time zero and after 5 minutes. The enzyme activity was measured in terms of NADPH left unoxidized (ϵ = 6.2 $mM^{-1}cm^{-1}$). Enzyme activities of ascorbate-glutathione cycle (APX, DHAR, GR) were expressed as units mg^{-1} protein as shown in legend under the figures. Enzyme activities were measured using a Beckman (Fullerton-CA) DU 7000 spectrophotometer. Protein was measured by the protein-dye binding assay (Bradford, 1976) method, using bovine serum albumin (BSA) as a standard.

Determination of Cd and Ca:

Dry samples were wet digested with 7 ml of 65% HNO_3, and 1 ml of 30% H_2O_2 at 200°C for 30 min using a closed vessel device using temperature control microwave system with high pressure segmented rotor [Cookbook for microwave digestion]. After dilution with deionized water, Cd and Ca were determined by Inductively Coupled Plasma (ICP-AES) with a standard method. Results were expressed as µg/g dry weight.

Statistical analysis:

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA).Values are shown as the mean (± SD) of three replicates. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test at a probability of $P \leq 0.05$.

RESULTS

Germination index and mitotic index:

The presence of Cd, alone or with added Ca, in the germinating medium resulted in reduced radicle length and showed necrotic symptoms 48h after both Cd treatments. The growth reduction involved cell proliferation, as demonstrated by the Cd-dependent inhibition of the mitotic (-41.9%) index (IM) occurring in the radicle apical meristem (Table 1). Although Ca induced Cd interfering uptake (Table 2) yet it depressed IM more negatively. Addition of Ca to Cd decreased IM by 55.2% compared with the control. Most cells were in resting stage. The dividing cells were swollen under both Cd treatments. Many cells were died, the nuclear shape of died cells were irregular and the chromatin shape was abnormal. The observed significant reduction in germination index (IG) was mainly due to diminished radicle length, but not due to great reduction in germination percentage, since all treatments were taken 12h water-

soaking initial phase. Germination index of seeds supplemented with 250 µM Cd was significantly decreased by 29.2%. Addition of Ca (500 µM) to Cd significantly decreased it by 26.0 % compared with the control (Table 1).

Table1. Mitotic index (IM, %) in radicle apex and germination index (IG, %) after 72 h of Cd (250 µM) and Cd+Ca (250 µM + 500 µM) exposure onset of germination

Treatments (µM)	Mitotic Index (%)	Germination Index (%)
Control	9.85	99.00^{a}±0.88
Cd 250	5.72	70.10^{b}±2.98
Cd 250+ Ca500	4.41	73.23^{b}±0.40

Data of IG represent the means (± SD) of ten replicates calculated as explained in Materials and Methods. Different letters indicate significant differences among the values (one-way ANOVA test).

Although IM of Cd+Ca treatment was decreased by 22.9% and also G% reduced by 3.9% compared with Cd treatment but its radicle length increased by 9.7%, therefore IG for both of them was non-significantly different. Because of membrane protection offered by Ca decreased ion leakage, causing more negative osmotic potential of radicle cells, so more water uptake and increased cell elongation.

Stress markers:

NAD(P)H oxidase,lipid Peroxidation and K^+ leakage:

NAD(P)H oxidase is considered to be oxidative stress-related enzyme. In radicle tissues, NAD(P)H oxidase activity was significantly stimulated after Cd exposure either alone or in combination with Ca compared with the control (Fig.1). Concentration of TBA- MDA, under Cd treatment, showed significant increase. It was more than two folds that of the control in radicle tissues. Addition of Ca led to significant reduction of MDA by 55.7% in radicle compared with that of Cd treatment (Fig. 1). Percentage of K^+ leaked from radicle tissues, due to Cd application, was found to be significant and represented more than two times that of control value. Addition of Ca to Cd decreased K^+ leakage significantly by 30.1% in comparison with Cd treatment (Fig. 1).

Glutathione concentration and ratio:

The glutathione pool [GSH plus GSSG] was significantly decreased in the radicle under Cd exposure. Combination of Ca with Cd increased its concentration by 9.1% compared with Cd treatment (Fig. 2A). The changes in the glutathione pool (on protein basis, data not shown) were mainly due to the decrease in the reduced form, i.e. GSH (due to sever reduction in GR activity) since, the levels of GSSG remained almost unaltered. On fresh weight basis, concentration of GSH (under 250 µM Cd) was significantly decreased by 51.0%, while that of GSSG was significantly increased by 34.7% in comparison with the control. The glutathione redox state was significantly lower, by 36.4% under both Cd treatments, than the control. The GSH /GSSG ratio of the control was 2.8 fold that of both treatments (Cd and Cd + Ca). In cotyledons, there was little decrease in total glutathione pool and GSSG due to Cd application, while addition of Ca to Cd increased total glutathione pool and GSSG significantly compared with Cd treatment. The redox state and GSH /GSSG ratio showed non-significant differences relative to the control (Fig. 2B).

Ascorbate concentration and ratio:

In the radicle, Cd induced a significant decrease of both the total ascorbate pool [Asc plus DHA] and Asc by 18.4 and 36.1%, respectively, while it caused an increase of DHA by 66.9% as compared with the control (Fig. 3A). Addition of Ca to Cd lowered the reduction of the previous parameters to become 7.9 and 27.3% due to enhanced DHAR activity, while it increased the oxidized form by 85.9%; therefore, a decrease in the ascorbate redox state was observed. In the cotyledons, both Asc and DHA of Cd treatment were decreased, while they increased on Ca addition over the control. On the other hand, no significant differences occurred in the redox state (Fig. 3B). The ratio between Asc/DHA of the control radicle was 2.6 folds that of either Cd or Cd +Ca treatment, while in cotyledons there were no significant differences, probably as a consequence of a lower rate of ROS production in the cotyledons. The Asc/DHA ratio in both organs, under both treatments, was more than unity (on the contrary to that for GSH) due to enhanced DHAR activity (Fig. 5). In the fitness of mitotic division, it was found that Asc concentration was higher in R than C under control and treatment conditions, Asc R/C ratio of the control reached 1.7, while DHA R/C ratio was 0.6 *i.e.* DHA concentration of R was much lesser than that of C. Cd caused an increase of DHA in R (low IM Table 1) than in C. DHA of R was the same as that of C on Ca addition.

Table2. Concentration of Ca and Cd (µgg^{-1}DW) in both radicles and cotyledons of germinating bean seeds subjected to 250 µM Cd and 250µM Cd +500 µM Ca treatments for 72h onset of germination

Treatments (µM)	Calcium concentration (µgg $^{-1}$DW)		Cadmium concentration (µgg $^{-1}$DW)	
	Radicle	Cotyledons	Radicle	Cotyledons
Control 0	1056.77^{c} ±38.99	1120.77^{a} ±135.88	0.27^{c} ±0.02	ND
Cd250	1161.87^{b} ±57.35	778.03^{b} ±14.90	74.57^{a} ±5.21	2.40^{a} ±0.05
Cd250+ Ca500	1276.63^{a} ±17.19	970.27^{ab} ±118.32	46.01^{b} ±3.42	1.94^{b} ±0.05

Data represent means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test). ND; non detectable concentration.

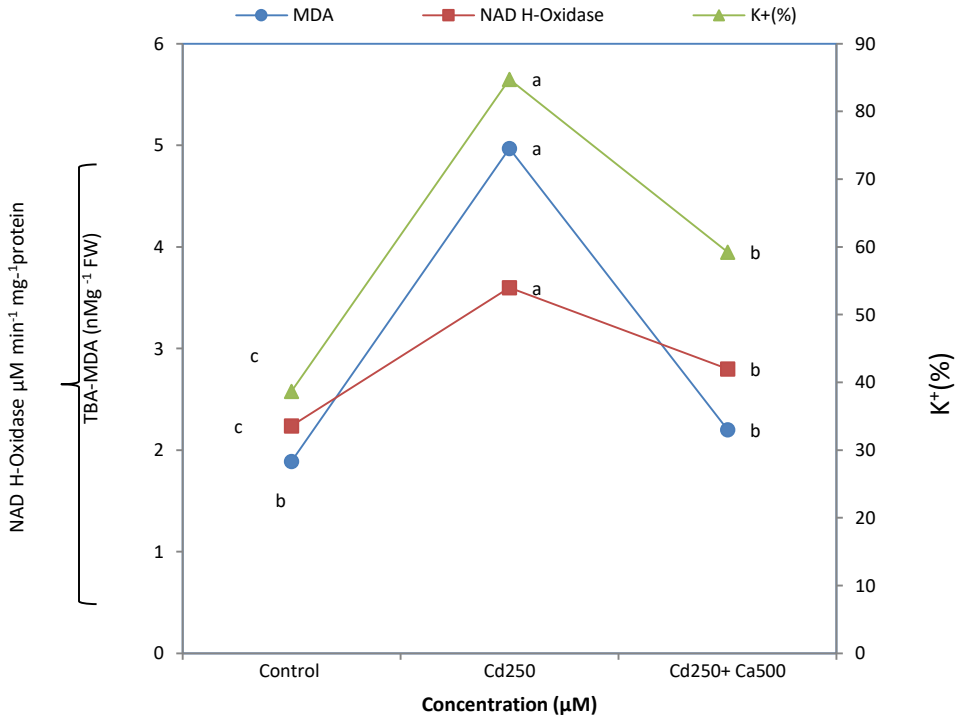
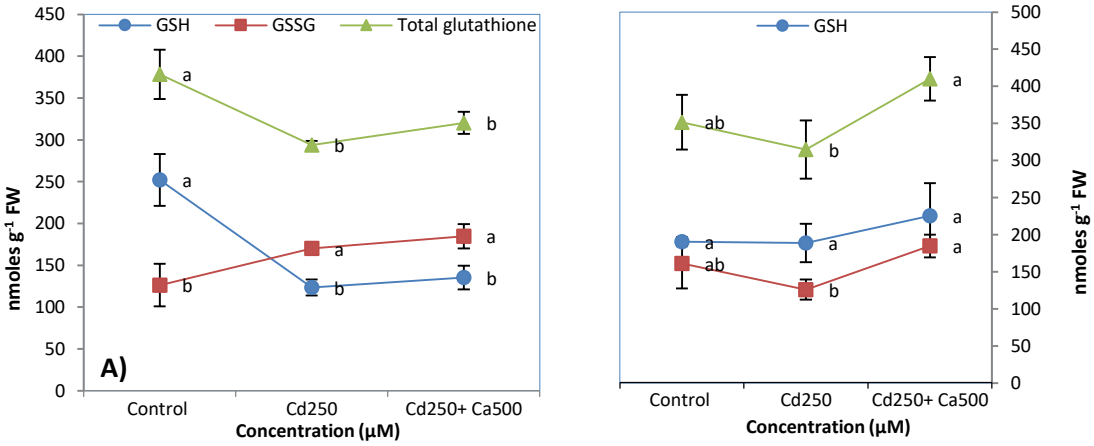


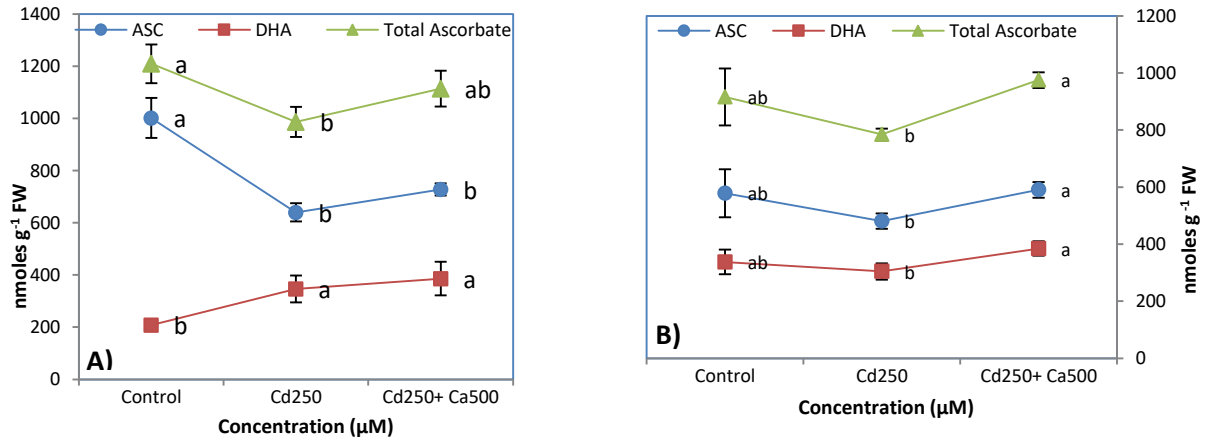
Fig.1. Stress markers; activity of NAD(P)H oxidase (µM min^{-1} mg^{-1} protein), TBA-MDA concentration (nM g^{-1} FW) and % of K^{+} leaked from radicle tissues of germinating bean seeds in response to Cd exposure (250 µM) and Cd+Ca (250 µM + 500 µM) treatments onset of germination. Data represent the means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test).



Redox state	0.66[a]±0.06	0.42[b]±0.03	0.42[b]±0.04
GSH/GSSG	2.06[a]±0.52	0.73[b]±0.08	0.74[b]±0.12

Redox state	0.55[a]±0.05	0.60[a]±0.01	0.55[a]±0.07
GSH/GSSG	1.22[a]±0.27	1.49[a]±0.06	1.24[a]±0.33

Fig. 2. Total glutathione pool, GSH, GSSG (nM g^{-1} FW), redox state and GSH/GSSG ratio in the radicles (A) and cotyledons (B) of germinating bean seeds grown for 72 h (onset of germination) in the presence of 250 µM Cd and 250 µM Cd+500 µM Ca treatments. The redox state was calculated as the GSH/GSH+GSSG ratio. Values represent the means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test).



Redox state	0.83[a]±0.02	0.65[b]±0.04	0.66[b]±0.04
ASC/DHA	4.87[a]±0.51	1.87[b]±0.31	1.93[b]±0.32

Redox state	0.63[a]±0.04	0.61[a]±0.02	0.61[a]±0.01
ASC/DHA	1.73[a]±0.29	1.58[a]±0.14	1.53[a]±0.08

Fig. 3. Total ascorbate pool, Asc, DHA (nM g^{-1} FW), redox state and Asc/DHA ratio in the radicles (A) and cotyledons (B) of germinating bean seeds grown for 72 h (onset of germination) in the presence of 250 µM Cd and 250 µM Cd+500 µM Ca treatments. The redox state was calculated as the Asc/ Asc+ DHA ratio. Values represent the means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test).

The percentage of increase in DHA of R/C ratio for both Cd and Cd+Ca treatments was 83.9 and 61.3%, respectively relative to the control. Total ascorbate pool was larger in the radicles than in the cotyledons, on both fresh weight and protein basis.

Activity of SOD and CAT:

Superoxide dismutase (SOD) activity of the control was 1.4 and 1.5 folds that of Cd and Cd+Ca treatments, respectively, in radicle tissues. In cotyledons, Cd decreased SOD activity by 12.7% in comparison with the control. On the other hand, the enzyme activity of Cd+Ca treatment increased significantly, it was1.6 times that of the control (Fig. 4A, B). Catalase activity in radicle tissues was significantly decreased due to Cd application either alone or in combination with Ca. The ratio of CAT activity between the control and Cd+Ca treatment was 1.7. In cotyledons, catalase activity of Cd+Ca treatment was increased by 7.5% over that of the control (Fig. 4A, B).

Enzyme Activities of Ascorbate- Glutathione Cycle:

When germinating seeds were exposed to Cd, ascorbate peroxidase (APX) activity reached to the control value and boosted it significantly by 18.9% in both radicle and cotyledons, respectively, while it reached to the control values on Ca addition in cotyledons (Fig. 5A, B). There was no significant difference in the activity of dehydroascorbate reductase (DHAR) in radicle tissues, while it was increased in cotyledons under both Cd treatments compared with the control (Fig. 5A, B). In cotyledons, the activity of DHAR, under Cd treatments, was much greater than that of the control. It was 1.7 fold the control value under Cd+Ca treatment. Therefore, there is no differences among Asc/DHA and redox state of treatments and the control (Fig. 3B). The behavior of both APx and DHAR may reflect the adaptability of the tissues to cope with the upcoming oxidative stress.

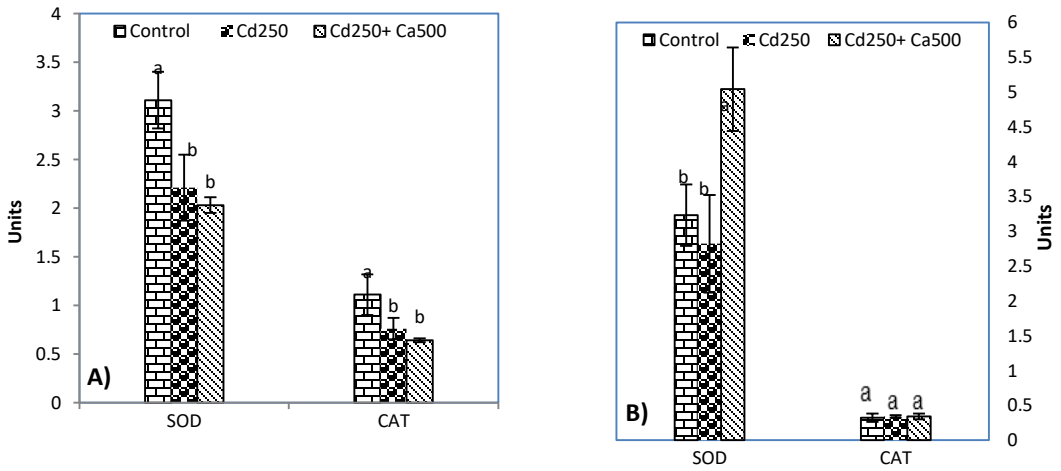


Fig. 4. Changes in the enzyme activities of SOD and CAT in the radicles (A) and cotyledons (B) of germinating bean seeds after 72 h of Cd treatments (onset of germination). Enzyme activities are expressed in units mg^{-1} protein. One unit of the SOD activity was defined as the amount of enzyme required for 50 % inhibition of the reduction of WST salt in comparison with the well lacking the enzyme. One enzyme unit for CAT was defined as the amount of enzyme required to oxidize 1 µM of H_2O_2 min^{-1} mg^{-1} protein. Data represent the means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test).

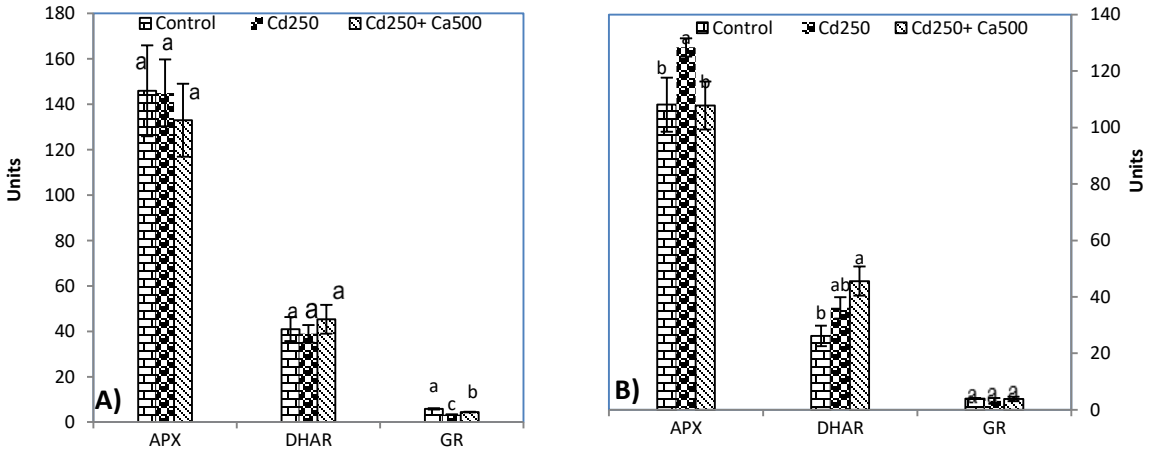


Fig. 5. Changes in the enzyme activities of ascorbate–glutathione cycle in the radicles (A) and cotyledons (B) of germinating bean seeds after 72 h of Cd treatments (onset of germination). Activities are expressed in units. APX, 1 unit=1 nmol Asc oxidized min^{-1} mg^{-1} protein; DHAR, 1 unit=1 nmol DHA reduced min^{-1} mg^{-1} protein; GR, 1 unit=1 nmol NADPH oxidized min^{-1} mg^{-1} protein. Data represent the means (± SD) of three replicates. Different letters represent values which are statistically different (by one-way ANOVA test).

Glutathione reductase (GR) activity, in radicle tissues, was significantly decreased under Cd treatment by 43.2% compared with the control, while it increased on Ca addition by 31.2% in comparison with Cd treatment. In cotyledons, GR activity showed non-significant differences (Fig. 5A, B).

Concentration of Cd and Ca:

Cadmium concentration was significantly increased in radicle tissues under both treatments (Cd and Cd+Ca) compared with the control. Application of Ca decreased Cd concentration in tissues of both radicle and cotyledons by 38.3 and 19.2%, respectively in comparison with Cd treatment on dry weight basis. Concentration of calcium in radicle tissues, of both treatments, was significantly higher than the control, while in cotyledons it was significantly decreased under Cd treatment relative to the control. Calcium concentration in cotyledons of the control was higher than that of radicle, while the reverse was found under Cd treatments either alone or in combination with Ca (Table 2). Release of cytosolic Ca due to higher mobilization of Ca from cotyledons to the embryonic axis to antagonize Cd, in addition to Ca taken-up from the external medium for competition with Cd in the latter case (Cd+ Ca treatment).

DISCUSSION

The most critical stages in the life cycle of higher plants are seed germination and seedling establishment. Germination starts with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley, 1997). The percentage of germination index (IG) was 70.8 % of the control value (Table 1) under Cd treatment. When the medium surrounding the seed was contaminated with Cd, delay in germination was often observed. This can be associated with several disorders in the event chain in metabolism of germinating seeds. Specially, seed germination and subsequent embryo growth are important stages of the plant life and highly sensitive to surrounding medium alterations, because the germinating seed is the first interface of material exchange between plant developmental cycle and the environment (Smiri, 2011). The interactions of Cd with decisive physiological functions in adult plants have been

widely investigated (Wagner, 1993). Cd disturbs the cellular metabolic processes by indirectly producing excessive ROS leading to oxidative stress. ROS is known to react with proteins, nucleic acids and lipids causing deleterious effects for various cellular processes. Its high affinity for sulfhydryl and oxygen containing groups results in blocking the essential functional groups of biomolecules (Møller et al., 2007). Plants react to abiotic stresses by the alterations in metabolic rates, protein turnover, osmolytes, membrane function, and gene expression. A number of studies have documented the production of ROS during the germination of different species (Bailly, 2004), which is considered a cause of stress that might affect the success of germination. Smiri (2011)suggested that alteration of redox and oxidative properties, in cotyledons and embryonic axis of pea seeds subjected to $CdCl_2$ (5 mM), is highly responsible for the decrease of germination success. The results reported here indicate that Cd negatively affected radicle growth of faba bean. The results also, show that Cd affected radicle growth by inhibiting cell division (Table 1), which matches with those previously obtained by Souguir et al., (2011)in faba bean. Concerning diminished mitotic index of radicle meristematic cells supplemented with Ca in addition to Cd, it was early when publications showed that low $[Ca^{2+}]i$ (<1.0 µM) was necessary to achieve microtubule polymerization *in vitro*, while higher concentrations blocked them. Spindle microtubules were shown to be sensitive to elevations in $[Ca^{2+}]i$ with depolymerization occurring when the concentration was raised to 1.0 µM or more (Zhang et al., 1992). Restriction of the $[Ca^{2+}]i$ was seen to affect progress through (Table 1) mitosis (Hepler, 2005). It has been reported by de Pinto et al. (1999)that a peak in Asc, but not GSH, levels coincided with a peak in the mitotic index of tobacco TBY-2 cells. Moreover, when the cells were enriched with Asc, a stimulation of cell division was occurred (Fig. 3A) whereas, when the cells were enriched with DHA, the mitotic index was reduced. They also, added that Asc/DHA pair has a specific regulatory role on cell division rather than acting as a general redox pair.

Cadmium has low redox potential and therefore it cannot participate in biological redox reactions as Cu does, but there are evidences that it could perform oxidative related disturbances, including lipid peroxidation (Vassilev and Lidon, 2011). This effect might be explained by the known high affinity of Cd ion to the functional groups of proteins, which may affect their functional properties (Schützendübel and Polle,2002). Cell membrane injury is associated with the production of ROS, which is indicated by the excess production of MDA. It is considered to be the general indicator of lipid peroxidation, which can be started by the redox-active metal ions themselves through the iron containing enzyme; lipoxygenase (Repetto et al., 2010). This membrane bound enzyme, which is known to produce free radicals, may be responsible for increased MDA level on Cd addition. Rahoui et al. (2010)revealed that Cd enhanced membrane damage and solute leakage in germinating pea seeds. These are in agreement with the presented results (Fig.1). A reduction in total phospholipids content of root plasma membrane as a result of degradation induced by Cd was reported. Plants supplied with Cd may have led to a lower mobility of lipid molecules. In addition, the looser binding of stigmasterol to phospholipid acyl-chains might alter the lipidic environment of intrinsic membrane enzymes, affecting the protein-phospholipids interaction, in a similar manner as pH affects solubility of aqueous soluble proteins. The changes in the content of phospholipids may have resulted in a loss of fluidity (Carruthers and Melchior, 1986). It may be suggested that when plant cells are not able to keep low tissue Cd level through efficient detoxifying systems, this may lead to a depletion of cell defence strategy and as consequence to Cd-provoked oxidative damages to important molecules; including lipids. As the lipids are main membrane constituent, so Cd disturbs membrane permeability allowing high level of K^+ leakage (Milone et al., 2003; Vassilev and Lidon, 2011), as shown in Fig. 1.

Abiotic stresses in legumes produce excess concentrations of ROS. The generation of ROS, beyond the antioxidant capacity of a biological system causes oxidative stress (Shigeoka and Maruta, 2014). Germinating seeds must adapt their metabolic activities and developmental processes to the dominating environmental conditions to become photoautotrophic before their nutrient reserves become exhausted. Since, ascorbate- glutathione pathway is the heart of the redox hub that occurs in many cell organelles, so it is studied exclusively. A decrease in Asc and GSH was observed in pea roots due to Cd exposure (Sandalio et al., 2001) as my results show (Figs. 2,3),while the opposite behavior was reported in durum wheat roots subjected to similar Cd concentrations. However, it is worth noting that

several differences in the effects of Cd on antioxidant metabolism have been reported in the literatures for different species and also for the same species under various Cd concentrations or when different organs were analyzed. In addition to the time of application as evident from this study, since the obtained results are contradictory with those reported by Souguir et al.(2013);the same plant organ, nearly the same Cd concentration and exposure duration, but the application, in this study, at the onset of germination. Their results revealed that GSH increased by 255% (12 day old faba root exposed to 200 µM Cd) and the GSH/GSSG ratio was 1.8 fold that of the control. In this work GSH/GSSG ratio of the control was 2.8 fold that of Cd (250 µM) treatment. Reduction of GSH has many causative factors: it is the precursor for phytochelatin synthesis for Cd chelation. The depletion of GSH could also contribute to oxidative stress by depressing the antioxidative response (Jozefczak et al., 2014). A decrease in GR activity can affect the GSH/GSSG ratio, but also decrease the ascorbate pool and its redox state (as in Figs. 2A, 3A)with an overall decrease in stress tolerance (Ding et al., 2009). Glutathione reductant for retrieving its reduced form; NAD(P)H may be greatly consumed by NAD(P)H oxidase (Fig. 1). Also, DHA is reduced by GSH-dependent DHAR, which is induced by various abiotic stresses. Finally, it may be utilized in H_2O_2 reduction by glutathione peroxidase, which is inducible under stress conditions.

The GSH/GSSG pair is also involved in protein folding by sulphydryl groups-disulphide bridges transition. It has been reported that a low GSH/GSSG ratio facilitates the folding and assembly of newly synthesized secretory proteins in the endoplasmic reticulum (Hwang et al., 1992). The GSH/GSSG ratio found in control bean radicle, in this study, was similar to that detected in the endoplasmic reticulum (around 2). It is also known that GSSG stimulates the rate of assembly of polymeric proteins by oxidizing specific cysteine residues. Glutathione plays an important role in the antioxidant defence system, since, it not only participates in the regeneration of ascorbate via DHAR (Fig. 5), but it can also react with $^{\cdot}OH$ and protect the protein thiol groups from the irreversible formation of intramolecular disulphide bonds. There are great evidences that either ROS or changes in the redox poise generated at sites of primary action of stressors could activate a coordinated response in other compartments (Fig.2B) to ensure a successful defence strategy. It has been suggested that the GSH/GSSG ratio, indicative of the cellular redox poise, may be involved in ROS perception. Foyer and Noctor (2011) suggested GSH /GSSG pair to the role of redox sensor rather than the Asc/DHA redox couple, since the redox status of the GSH pool is influenced more intensively (as the presented case, Figs. 2, 3) by the elevated ROS formation. Moreover, the bulk of the GSSG is localized in the cytosol, but the considerable amount of DHA is probably localized in the apoplast, hence plant cells can maintain low cellular GSH/GSSG and relatively high Asc/DHA ratios at the same time. The availability of Asc and GSH and changes in their redox state also affect gene expression (Catani et al., 2001).

The recycling of ascorbate, similarly to its biosynthesis, can provide the appropriate level of ascorbate for the stressed cells. The increase in DHAR expression increased foliar and kernel ascorbic acid levels 2- to 4-fold and a significantly increased ascorbate redox state were observed in tobacco and maize(Chen et al., 2003). Under different stresses, transgenic plants showed a larger ascorbate pool size and lower membrane damage compared with controls. Transgenic plants over-expressing MDHAR and/or DHAR showed enhanced tolerance to various stresses (Wang et al., 2010). These results suggested that increasing the plant ascorbate content through enhanced ascorbate recycling could limit the harmful effects of environmental oxidative stress. Cellular antioxidants may also play an important role in inducing resistance in plants to metals by protecting labile macromolecules against attack by ROS (as the case in cotyledons, Fig. 3B, no change in Asc redox state due to enhanced DHAR activity) which lead to oxidative stress (Belkadhi et al.,2016).

NADPH oxidase transfers electrons from NADPH to O_2 to form superoxide radical ($O^{\cdot}{}_2^-$), followed by the dismutation of $O^{\cdot}{}_2^-$ to H_2O_2. Smiri (2011) reported that Cd caused a significant consumption of reduced nicotinamide, as evidenced by the increase in $NADP^+$ balance, probably by the stimulation of enzymatic oxidation *via* NADPH oxidase activity (Fig.1). It is considered to be oxidative stress-related enzyme and its activity was strongly stimulated after Cd exposure. ROS might be actively produced by NADPH oxidases (Bielen et al.,2013). Oxidative stress leads to apoptosis; programmed cell death (PCD), which is initiated and propagated through the generation of ROS. Low doses induce antioxidative enzymes; however, having reached

a certain threshold, concentration of ROS activates a signal transduction pathway, which results in PCD (Dickman et al., 2017).Superoxide dismutase (SOD) catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen. Therefore, the activity of this enzyme determines the relative proportions of the two constituents of the Haber-Weiss reaction that generates hydroxyl radicals. It has been assumed that SOD has a central role in the defence against oxidative stress (Scandalias, 1993). A decrease in SOD activity (Fig. 4A) after prolonged exposure to 40 ppm Cd was observed John et al., (2007), which may be due to the enhanced level of H_2O_2, as reported previously in oat leaves under Cu stress. Catalase is a heme-containing enzyme that catalyzes the dismutation of H_2O_2 into water and oxygen. The enzyme is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle and purine catabolism. The presented results are in agreement with those of Shaw (1995), describing a decrease of catalase activity and an increase of ascorbate peroxidase activity in *Phaseolus aureus* treated with Cd. A significant reduction of CAT activity in pea leaves with increasing Cd concentration was reported(Sandalio et al., 2001). Decline in CAT activity, a general response to many stresses, which may be due to inhibition of enzyme synthesis or change in assembly of its subunits or attack by peroxisomal proteases (Shim et al., 2003). For the most part these effects are thought to be linked to Cd extreme capability to bind sulfhydryl groups of proteins, leading to enzyme inactivation. Moreover, Cd accumulation induces oxidative stress as evidenced by the formation of ROS (Bielen et al.,2013). However, since Cd is not a redox-active metal, oxidative stress could result from both interferences with GSH metabolism and redox-active metal displacement from proteins (Schützendübel and Polle,2002).

Ascorbate peroxidase (APx) is always very active in dividing cells and tissues undergoing differentiation. Ascorbate supplied by means of the reduction of its oxidized forms is necessary for the activation of the Asc dependent metabolic pathway at the beginning of germination (Fig. 5A), since a lag of several hours is required for the start of Asc biosynthesis (Tommasi et al., 2001). APx is a central enzyme of Asc-GSH cycle, and plays an essential role in the control of intracellular ROS levels. It uses two molecules of Asc to reduce $H_2O_2$to water with a concomitant generation of two molecules of MDHA. APx is regulated by redox signals and H_2O_2 (Patterson and Poulos, 1995). It was indicated that phytotoxic concentrations of Cd (10-100 µM) in the soil can cause inhibition of several peroxidases, but induction of peroxidases is a general response of higher plants, which is considered to play a significant role in stress metabolism. Dehydroascorbate reduced by GSH-dependent DHAR, which is induced by various abiotic stresses and is thought to be critical for stress tolerance (Jozefczak et al., 2015). Oxidative stress induced inhibition in GR activity has been observed in transgenic tobacco plants. A significant depression in GR activity (Fig. 5A) after exposure to sub-lethal Cd concentration (250 µM) in this study matches with the previous results. GR contains a highly conserved disulphide bridge between Cys 76 and Cys 81, which may undergo cleavage by heavy metals at toxic concentrations (Creissen et al., 2000). Varying responses of Cd induced oxidative stress are probably related both to levels of Cd supplied and to concentration of thiolic groups already present or induced by the metal. Thiols possess strong antioxidative properties, and consequently counteract oxidative stress (Jozefczak et al., 2014).

It has been known that Ca^{2+}, by binding to phospholipids, stabilizes lipid bilayers and thus provides structural and functional integrity to cellular membranes, so it controls membrane permeability (Hepler, 2005), as shown from the data presented here (Fig. 1). Cd, as well as other nonessential metal ions, is thought to be taken up by plants *via* cation transport systems normally involved in the uptake of essential elements, such as members of zinc and iron-regulated transporter-like proteins (ZIP family) or Ca^{2+} channels and transporters (Clemens, 2006). Calcium amendment significantly lowered tissue Cd concentration (Table 2). This suggests that Ca enrichment decreases Cd accumulation through displacement of cell surface from toxic cations. High concentration of Ca^{2+} around plasma membrane reduces cell-surface negativity and harmfulness of cationic toxicants and/or reduces the uptake of Cd *via* calcium channels to mimic Ca (Suzuki, 2005). Calcium signaling features in response to a number of abiotic stress factors were reported. Excess heavy metals modify the stability of Ca channels, thus increasing calcium flux into the cell. Intracellular Ca is a secondary messenger, which interacts with calmodulin to propagate the signal and ultimately to regulate downstream genes involved in heavy metal transport, metabolism, and tolerance (Yang and

Poovaiah, 2003). Higher intracellular Ca levels were observed in plants exposed to Cd (Table 2) inducing adaptive mechanisms that alleviate the toxic effects of heavy metal stress. Wang and Song (2009) reported that application of 5 mM $CaCl_2$ reversed the Cd effects on the activity of SOD, catalase, glutathione peroxidase and APX and reversed the Cd-induced decrease in fresh mass as well as the changes in lipid peroxidation in *Trifolium repens* (as in Figs. 4B, 5B& 1). Ca treatment had two positive effects on citrus physiology: it reduced Cd^{+2} uptake into roots and also increased GSH content. The capacity to maintain optimum GSH levels to feed phytochelatin biosynthesis could also be an important factor in stress tolerance (López-Climent et al., 2014). Cd may exert its toxicity through Ca signaling accompanied by mitochondrial depolarization (Shih et al., 2005).

CONCLUSION

In conclusion, a general shift of the cellular redox poise towards the oxidative state was found to affect radicle more than cotyledons severely. Cd caused a depletion of GSH and to a lesser extent Asc, and an inhibition of antioxidative enzyme activities, especially that of GR. Assessment of antioxidative capacities by metabolic modeling suggest that the reported diminution of antioxidants was sufficient to cause ROS accumulation. The depletion of GSH is a critical step in Cd sensitivity. Available data suggest that Cd, when not swiftly detoxified enough may trigger, *via* the disturbance of the redox control of the cell, a sequence of reactions leading to reduced mitosis, growth inhibition, diminished ratios of GSH/GSSG and Asc/DHA, stimulation of secondary metabolism, lignification, and finally cell death. The negative role of Ca in ameliorating redox poise of the cell may be due to the involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of seed germination.

CONFLICT OF INTEREST

The author declared that present study was performed in absence of any conflict of interest", since this work was done by a single author; Dr. Zeinab Ahmed Khidr (Assistant Prof. of Plant Physiology. Faculty of Science, Al-Azhar Univ.).

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AUTHOR CONTRIBUTIONS

ZAKh designed and performed the experiment, wrote and reviewed the manuscript. I approved the final version after correction.

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