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The Responses of Embryos to Cadmium Induced Oxidative Stress in Germinating Pea Seeds

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ABSTRACT

The present study showed the evidence of the generation of an oxidative stress in the growing embryos of germinating pea (*Pisum sativum* L. cv. douce province) seeds, after exposure to 200 µM cadmium chloride ($CdCl_2$). Hence, we focused on understanding Cd induced oxidative stress, its consequences and the biological significance of some enzymatic and non-enzymatic antioxidants as potential selection criteria for improving tolerance to oxidative stress have been discussed. The effects of Cd on some indicators of oxidative stress were analyzed in order to study relationship between metal toxicity, oxidative stress and detoxification responses. Obtained results revealed a protection of embryonic axes growth and metabolism against the heavy metal up to day 6 of germination, beyond then severe metabolic disturbances have been observed leading to oxidative injury to proteins and marked disruption of the structural integrity of membranes and cellular homeostasis. This might be associated to the non-efficiency of the antioxidant defense systems at day 9, which resulted in the delay in embryonic growth.

INTRODUCTION

In recent years, heavy metals toxicity has become a problem of both agricultural and environmental significance owing to mining, excess of use of fertilizers and discharge of untreated urban, agricultural and industrial residues [1], which leads to widespread soil contamination. Seed germination and subsequent embryo growth are important stages of the plant life and highly sensitive to surrounding medium fluctuations, because the germinating seed is the first interface of material exchange between plant development cycle and environment [2]. The toxic trace pollutants have been shown to interfere with morphological, physical and metabolic irregularities in seed germination and plant growth [3-8]. In plant cells, they can shift the intracellular redox balance toward an oxidizing state [9-12] and disrupt the prooxidants/antioxidants equilibrium, tilting the balance in favor of the latter, inducing reactive oxygen species (ROS), and directly reacting with functioning cellular macromolecules and organelles [13-15]. One of the most common antioxidative protein modifications is the formation of carbonyl derivatives, in which the amino-acid side chains, mainly histidine, arginine, lysine, proline, threonine and tryptophan residues, are converted by the action of ROS to aldehyde or keto groups [16].

Plants employ various inherent and extrinsic defense strategies for tolerance or detoxification whenever confronted with the stressful condition caused heavy metals. Largely, plants can be classified into four groups: metal-sensitive species, metal-resistant excluder species, metal-tolerant non-hyperaccumulator species, and metal-hypertolerant hyper-accumulator species, each having different molecular mechanisms to accomplish their resistance/tolerance to metal stress or reduce the negative consequences of metal toxicity. One of the strategies adopted is the avoidance to preclude the onset of stress via restricting metal uptake from soil or excluding it and preventing metal entry into plant. This can be achieved by some mechanisms such as immobilization of metals by mycorrhizal association, metal sequestration, or complexation by exuding organic compounds from root. At next stage, if these strategies fail and heavy metals manage to enter inside plant tissues, tolerance mechanisms for detoxification are activated which include metal sequestration and compartmentalization in various intracellular compartments (e.g., vacuole), intracellular complexation or chelation of metal ions by organic acids, polysaccharides, phytochelatins (PCs), and metallothioneins (MTs). If all these measures prove futile and plants become overwhelmed with toxicity of heavy metal, activation of antioxidant defense mechanisms is pursued [6,11,13,17-19]. These involve; (i) antioxidative enzymes, i.g. superoxide dismutase (SOD; EC 1.15.1.1) [20], glutathione peroxidases (GPX; EC 1.11.1.9), guaïcol peroxidase (G-POX; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6), (ii) enzymes

of the ascorbate-glutathione cycle [21] and (iii) non enzymatic antioxidants molecules like ascorbate (AsA), glutathione (GSH), carotenoids, alkaloids, tocopherols, proline, and phenolic compounds (flavonoids, tannins, and lignin) that act as free radicals scavengers [22,23]. Abiotic stresses may also induce the emission of multiple biogenic volatile organic compounds (BVOCs) such as terpenes, oxylipins, methanol, ethanol, formaldehyde, acetaldehyde, etc. Although many studies have investigated heavy metals toxicity in plants, there are still many uncertainties and gaps in our understanding of mechanism of ROS generation and the potential strategies to enhance oxidative stress-tolerance in plants. In current work, the various detrimental consequences of pea seeds exposure to cadmium (Cd) stress were discussed. This ranged from physiological manifestation of Cd toxicity on the main organs of seeds; the embryonic axes, to intracellular detoxification.

MATERIALS AND METHODS

Germination and cadmium treatment

Pea (*Pisum sativum* L. cv. douce province) seeds were surface-sterilized with 2% of sodium hypochlorite for 10 min and then germinated at 25°C in the dark over two sheets of filter paper moistened continuously with the same volume of distilled water or 200 µM $CdCl_2$. At appropriate intervals of 0, 3, 6 and 9 days, embryonic axes were sampled for the assays.

The 200 µM $CdCl_2$ concentration was chosen on the basis of preliminary experiments in which this concentration was demonstrated to cause 50% of inhibition of growth parameters (Data not shown).

H_2O_2 measurement

H_2O_2 levels were determined according to Sergiev et al. [24]. Extraction was performed with 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12000 × g for 15 min at 4°C. The absorbance of the supernatant was measured at 390 nm, after addition of 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (KI).

Malondialdehyde determination

Samples (about 3 g) were homogenized in 20 mM Tris-HCl (pH 7.4; 1:3, sample fresh weight/buffer volume), centrifuged at 3000 × g for 20 min, and then derivatized in a 1 mL reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1, v/v), HCl 32%, 100 mL water and an equal volume of sample or 0.6 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl (pH 7.4). After 40 min of incubation at 45°C, samples were cooled on ice, centrifuged at 15000 × g for 10 min and the absorbance of the supernatant was recorded at 586 nm. Levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol mg-1 protein.

Carbonyl and thiol groups assay

Carbonyl groups were extracted in 10 mM sodium phosphate buffer pH 7.4 containing 1 mM EDTA, 2 mM DTT, 0.2% triton X-100 (v/v) and 1 mM PMSF. After centrifugation (27000 × g /20 min /4°C), the supernatant was used for CO assays. Quantification of protein carbonyl groups was carried out according to the classical approach of Reznick and Packer [25] using a spectrophotometric DNPH method. The mixture of protein extract and dinitrophenylhydrazine (10 mM, prepared in 2 N HCl) was allowed to stand in the dark at room temperature for 1 h with continuous vortexing, and then precipitated with cold trichloroacetic acid (20% final concentration) and centrifuged at 20000 × g for 15 min. The protein pellet was washed with 20% trichloroacetic acid, and then three times with ethanol/acetic acid (v/v). Samples were then resuspended in 6 M guanidine hydrochloride (dissolved in 2 N HCl) and incubated at 40°C for 30 min with vortex mixing. Carbonyl content was determined from the absorbance at 480 nm (ε=22000 M^{-1} cm^{-1}) as described by Levine et al. [26]. The protein-carbonyl content was expressed as nmol mg^{-1} protein.

Total protein thiols were assayed according to Ellman [27]. The reduction of 5.50-dithiobis (2-nitrobenzoic acid), (ε=13600 M^{-1} cm^{-1}), was followed by measuring the increase in the absorbance at 412 nm.

Proline assay

Proline content was determined according to the modified method of Bates et al. [28]. Samples were homogenized in a mortar and pestle with 10 ml sulphosalicylic acid (3%, w/v), then filtered through Whatman 2 filter paper and centrifuged at 18 000 × g for 15 min. 2 ml of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 hour at 100°C and then allowed to cool to room temperature. Then 4 ml of toluene were added to the reaction and mixed on a vortex mixer for 15-20 seconds. The test tubes were allowed to stand for at least 10 min to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at a wave length of 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve.

Enzymatic activities assay

Catalase, CAT (EC 1.11.1.6), activity was measured according to Aebi [29] and estimated by monitoring the decrease in absorbance of H_2O_2 reduction at 240 nm (ε = 36 × 10^{-6} $M^{-1}.cm^{-1}$). Superoxide dismutase, SOD (EC 1.15.1.1), activity was measured according to Mishra and Fridovich [30], using 1.88 U mL^{-1} catalase. SOD activity was estimated at 490 nm, using epinephrine as

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standard. Ascorbate peroxidase, APX (EC 1.11.1.11), activity was measured according to Nakano and Asada [31] by monitoring the decrease in the absorbance of ascorbate at 290 nm (ε = 2.8 × 10^{-3} $M^{-1}.cm^{-1}$). Guaïcol peroxidase, G-POX (EC 1.11.1.7), activity was measured according to Fielding and Hall [32] by monitoring the increase in absorbance of tetraguaiacol at 470 nm (ε = 26.6 $M^{-1}.cm^{-1}$). Glutathione peroxidase, GPX (EC 1.11.1.9), activity was measured according to Nagalashmi and Prasad [33], using 1 mM glutathione reduced (GSH), 2.5 mM H_2O_2, 0.5 mM NADPH and 1 U GR (100-300 units/mg protein, SİGMA). The oxidation of NADPH was evaluated by measuring the decrease in the absorbance at 340 nm (ε = 6.22 × 10^3 $M^{-1}.cm^{-1}$, Navrot et al. [34]).

Protein determination

Protein concentration in enzyme extract was evaluated by the method of Bradford [35], using bovine serum albumin as standard protein.

Statistical analysis

The experiment was repeated at least twice. The presented data are from representative experiment. The mean values ± SE were compared for significance of differences at $p<0.05$ using ANOVA test followed by T-test student's analysis.

RESULTS AND DISCUSSION

Effect of cadmium of embryo growth

The growth of pea seeds was analyzed during germination time in the absence or presence of Cd stress. **Figure 1** showed reduced length of the embryonic axes of Cd-treated seeds, as compared to controls. This delay in growth exceeded two folds less then controls after 6 and 9 days. Indeed, it has been reported that under excess heavy metal exposure, plants display reduced biomass, inhibited root growth, and morphological alterations, often leading to plant death at excessive exposures [36]. In order to shed light on this point, we interested on studying the cellular metal homeostasis in the growing embryonic axes, which includes regulation of the metal-induced reactive oxygen species (ROS) signaling pathway.

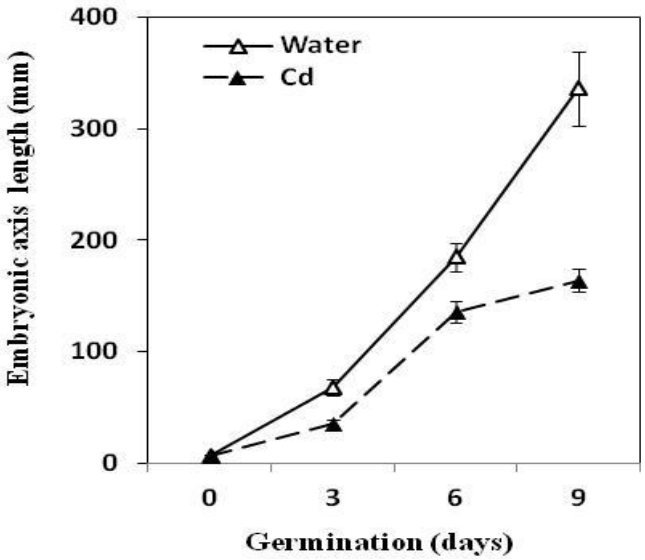

Figure 1. Embryonic axis length of pea (Pisum sativum L. cv. douce province) seeds during germination in the presence of distilled water or 200 µM $CdCl_2$. Values are means of four measurements (± SE). Letters a, b and c refer to $p< 0.05$, $p< 0.01$ and $p< 0.001$, respectively.

Changes in the oxidative stress parameters under cadmium stress

Figure 2 revealed increased levels of hydrogen peroxide H_2O_2 levels in Cd exposed seeds, which showed the evidence of oxidative processes generation, which agrees with other reports in many heavy metal-stressed-species [6,19,37]. This finding can cause oxidative stress via disturbing the equilibrium between pro-oxidant and antioxidant homeostasis within the plant cells. Hence, the mechanism by which Cd seems to operate consists in a rather indirect metal effect, presumably by the induction of the generation of oxidative stress. This may, therefore, result in multiple deteriorative disorders and cellular disregulation, mainly oxidation of proteins and lipids, oxidative DNA attack, redox imbalance and denature of cell structure and membrane, ultimately resulting in the activation of programmed cell death (PCD) pathways. In addition, Cd is non-redox active metals indirectly inflict oxidative stress via multiple mechanisms including glutathione depletion, binding to sulfhydryl groups of proteins, inhibiting antioxidative enzymes, or inducing ROS-producing enzymes like NADPH oxidases. Here, our results showed that Cd treatment increased the levels of lipid peroxidation products MDA after 9 days in Cd germination **(Figure 2)**, which may resulted in the disruption of the structural integrity of membranes. Similar results have been reported by other authors in many heavy metals stressed species

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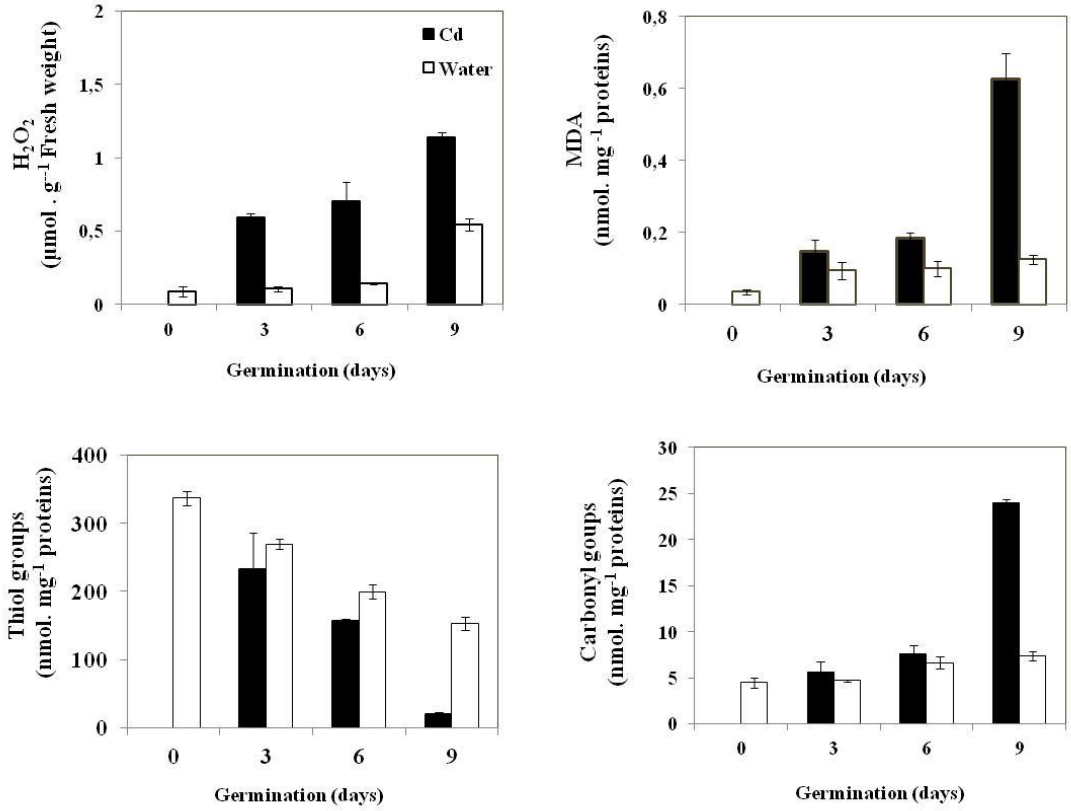

Figure 2. Levels of hydrogen peroxide (H_2O_2), malondiadehyde (MDA), thiol groups and carbonyl groups in pea (*Pisum sativum* L. cv. douce province) seeds during germination in the presence of distilled water or 200 µM $CdCl_2$. Values are means of four measurements (± SE). Letters a, b and c refer to $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

[2,6,12,19]. Besides, proteins have also been target of ROS, which has been confirmed by the modifications in protein status. Indeed, an increase in protein carbonyl groups was observed, concomitant with a significant decrease in reduced SH pools at 9 day **(Figure 2)**; which may lead to the depletion of thiol groups, as well as to sustained and irreversible protein damage, followed by loss of function. Similar results were reported by other authors working with different heavy metals [12,26, 29,38]. Furthermore, **Figure 3** showed a marked enhancement of the antioxidant enzymes activities SOD, CAT and peroxidases (APX, GPX and G-POX) in the presence of Cd, after 3 and 6 days, thus providing a sufficiently efficient antioxidant defense mechanism against heavy metal-induced oxidative stress. However, after 9 days, the activities of the different antioxidative enzymes decreased as compared to respective controls **(Figure 3)**. Similar elevation of the production of ROS scavenging antioxidant enzymes, providing a sufficiently efficient antioxidant defense mechanism against heavy metal-induced oxidative stress were reported by other authors [6,14,18,19,37,39,40].

Furthermore, the biosynthesis of diverse cellular biomolecules is the primary way to tolerate or neutralize metal toxicity. This was clearly evident by the induction of a tremendous free-proline accumulation in Cd treated embryos, especially at 3 and 6 days, as compared with controls **(Figure 4)**. Indeed, it has been reported the multi-functionality of proline in aiding plants to tolerate heavy metals since it can exhibit both chelating and antioxidant-related activities. It is also important to point out, here, that control seeds exhibited elevation in proline content as well, which could be related to other roles played by proline that include promoting embryo and seed development [41]. In our work, this production was much pronounced in the presence of Cd, which agrees with other reports as response to tensions caused by a wide range of biotic and abiotic stressors such as excessive salinity, drought, increased solar ultraviolet radiation, heavy metals, and oxidative stress. Besides, it has been reported that proline increases the resistance of plants to heavy metals toxicity, by acting as a metal chelator, proteins, macromolecules and organelles stabilizer, as well as enzymes protector from denaturation [42]. Rastgoo and Alemzadeh [41] studying effects of heavy metals (Cd, Co, Pb, and Ag) on Gouan (Aeluropus littoralis) reported a maximum proline accumulation occurring under Cd stress. On the other hand, the suppression of proline accumulation occurred at day 9, in comparison with days 3 and 6, may suggest that proline content increased as the amount of Cd increased beyond a certain threshold and to a specific level. Indeed, it has been reported that Cd is a non-essential heavy metal that can evoke a strong response of plant cells even when only applied at low concentrations [43,44]. Hence, when the applied Cd concentration is below the limit, presumably up to day 6, pea seedlings seem to be tolerant and survive. However, when the encountered concentration exceeds this limit, pea embryonic axes become sensitive and display Cd-toxicity, which was

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mainly revealed beyond day 6 by; (i) the marked delay in growth **(Figure 1)**, (ii) the elevated ROS-induced cells and molecules damages **(Figure 2)**, and (iii) the non-efficient antioxidant enzymatic and non-enzymatic defenses **(Figures 3 and 4)**. Hence, the accumulation of proline and

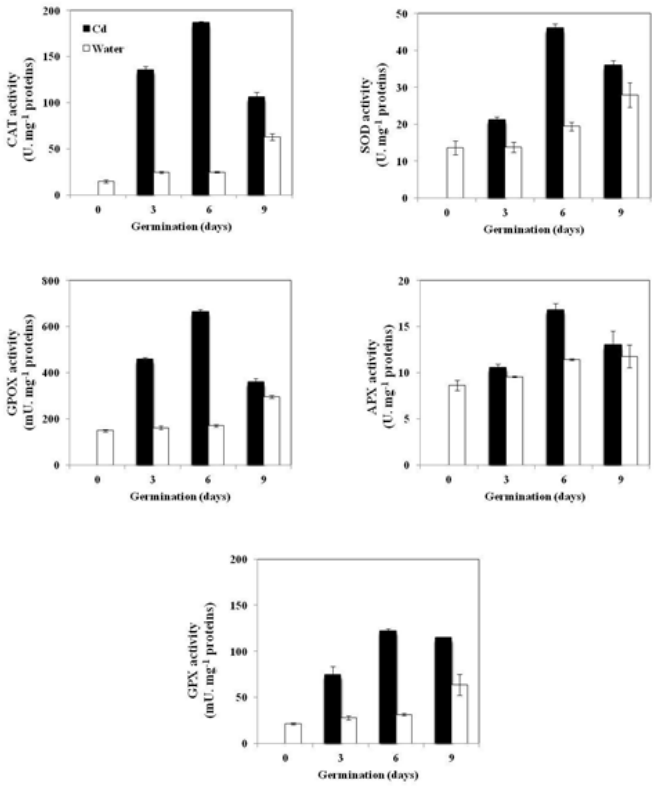

Figure 3. Antioxidative enzymes activities of CAT, SOD, GPOX, APX and GPX in pea (Pisum sativum L. cv. douce province) seeds during germination in the presence of distilled water or 200 µM CdCl2. Values are means of four measurements (± SE). Letters a, b and c refer to p<0.05, p<0.01 and p<0.001, respectively.

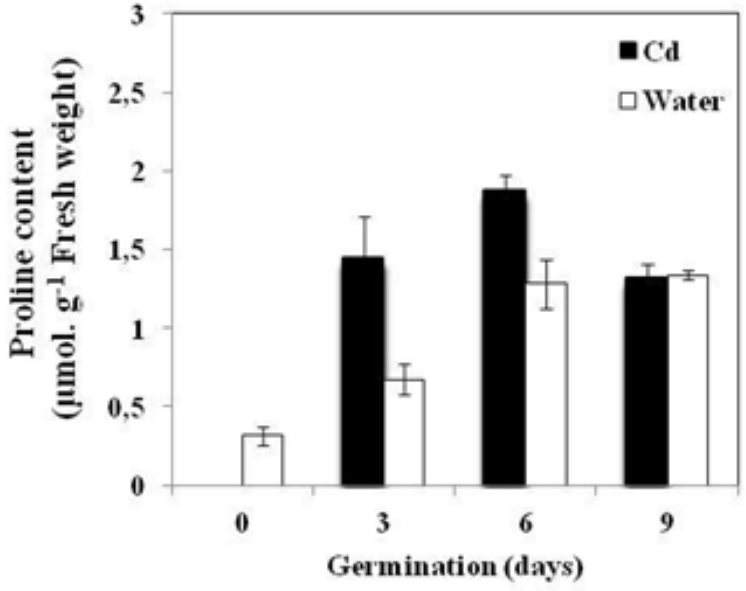

Figure 4. Levels of proline in pea (Pisum sativum L. cv. douce province) seeds during germination in the presence of distilled water or 200 µM $CdCl_2$. Values are means of four measurements (± SE). Letters a, b and c refer to p<0.05, p<0.01 and p<0.001, respectively.

the enhancement of the antioxidant enzymes during first days of Cd contamination can be considered as indicators of tolerance to the heavy metal stress, while followed by declined tolerance of the growing seedlings and increased Cd toxicity at day 9, which could be explained by the decrease of the efficiency of the defense systems. However, this direct link between metal toxicity and

activation/inhibition of responses against oxidative stress still require further investigations at a molecular level.

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