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Research Article

Cadmium Stress Induced Changes in Antioxidant Enzymes, Lipid Peroxidation and Hydrogen Peroxide Contents in Barley Seedlings

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Abstract: Cadmium pollution is a problem of increasing significance for ecological, nutritional and environmental reasons. Different plant species and varieties show a wide range of plasticity in Cadmium tolerance, from a high degree of sensitivity to the hyper-accumulating phenotype of some tolerant plants. To avoid Cadmium toxicity, plants adopt various defense strategies. The present study was undertaken to assess and investigate the antioxidant responses of barley (*Hordeum vulgare* L.) to cadmium treatment, seedlings of barley were grown in increasing concentrations of $CdCl_2$ ranging from 25-100 µM, for up 14 days in a hydroponic system. The results showed that $CdCl_2$ reduce pigment content and caused oxidative damage as characterized by increased total soluble protein, Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) contents. Under cadmium stress, the activities of antioxidative enzymes, including Ascorbate Peroxidase (APX), Peroxidase (POD) and Catalase (CAT) were increased considerably in plant tissues. The present results allow us to conclude that the barley plants showed a negative response to cadmium toxicity. The physiological and biochemical process in plants was significantly affected by stress of $CdCl_2$. To deal with the cadmium induced oxidative stress, barley plants activated antioxidant enzymes to diminish the Reactive Oxygen Species (ROS).

Keywords: Antioxidant enzyme, cadmium, *Hordeum vulgare*, oxidative stress, reactive oxygen species, tolerance

INTRODUCTION

Heavy metal contamination in soil mainly due to industrial emission, the application of sewage sludge and phosphate fertilizers containing Cadmium (Davis, 1984), has become a serious problem in crop and vegetable production. It is widely recognized that cadmium taken up by plants is the main source of cadmium accumulation in food (López-Millán *et al*., 2009). Cadmium can be easily absorbed by plant roots and transported to shoots. Roots are likely to be firstly affected by heavy metals since much more metal ions are accumulated in roots than shoots (Sanita di Toppi and Gabbrielli, 1999). Numerous studies have indicated that cadmium causes nutrient deficiency (Boulila *et al*., 2006; López-Millán *et al*., 2009) and induces inhibition of chlorophyll biosynthesis and a decline in the photosynthetic rate (Tukaj *et al*., 2007; Lopez-Millan *et al*., 2009). Cadmium toxicity also causes oxidative damages, such as cell membrane lipids and protein damages to plants, directly or indirectly via Reactive Oxygen Species (ROS) formation (Lesser, 2006). ROS can be quenched by the induction of enzymatic defense systems to alleviate the oxidative damage in Cadmium stressed plants (Wu *et al*., 2003).

Cadmium is a toxic element traces causing serious problems in cereal crops (Prasad, 1995). Among the oldest and most important crops, we expect Barley (*Hordeum vulgare* L.). It now ranks fifth among all cultures in the production of cereals in the world behind corn (*Zea mays* L.), wheat (*Triticum* spp.), Rice (*Oryza sativa* L.) and soybeans. The importance of barley stems from its ability to grow and be productive in marginal environments, which are often, characterized by drought, low temperatures, conditions of high salinity (Maas and Hoffman, 1997) and pollution of heavy metals (Dionisio-Sese and Tobita, 1998).

Therefore, the barley plants (*Hordeum vulgare* cv. Saida) were chosen as a biological model in this study. The objective of this study was to evaluate the responses of the defense system and the tolerance of barley cultivar Saida under stress induced by cadmium.

MATERIALS AND METHODS

Plant material and treatments: Seeds of "*Hordeum vulgare* cv. Saida" were provided by the Algerian

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Office Inter Cereals (AOIC) El Hadjar Annaba, Algeria. Barley seeds were sterilized by 5% sodium hypochloride solution for 10 min and then rinsed thoroughly with distilled water, submerged in deionized water in the dark overnight and germinated on filter paper in dishes petri. Twelve-days-old uniform seedlings (second leaf stage) were transplanted on to 3 L pots. The composition on the basic nutrient solution consisted of the following compounds (mg/L): $(NH_4)_2SO_4$ 48.2, $MgSO_4$ 65.9, K_2SO_4 15.9, KNO_3 18.5, $Ca(NO_3)_2$ 59.9, KH_2PO_4 24.8, Fe citrate 6.8, $MnCl_2 \cdot 4H_2O$ 0.9, $ZnSO_4 \cdot 7H_2O$ 0.11, $CuSO_4 \cdot 5H_2O$ 0.04, H_3BO_3 2.9 and H_2MoO_4 0.01. Different concentrations of $CdCl_2$ (25, 50, 75 and 100 µM, respectively) were added to the nutrient solution. The pH of the culture solution in each pot was adjusted every other day with 1 M HCl or NaOH as required. The solution was continuously aerated with an air pump and renewed every 4 days.

Determination of chlorophyll and carotenoid contents: The concentrations of chlorophyll a, b and carotenoids (mg/g FW) were evaluated by adopting the method given by Arnon (1949). The absorbance was measured at 663, 645 and 470 nm, respectively. The concentration of pigments was calculated according to the formulas of Lichtenthaler and Wellburn (1985).

Lipid peroxidation and hydrogen peroxide determinations: The level of lipid peroxidation in plant tissues was expressed as 2-Thiobarbituric Acid (TBA) reactive metabolites, mainly Malondialdehyde (MDA) and was determined according to Hodges *et al*. (1999). Fresh samples (leaves and roots) of around 0.5 g were homogenized in 4.0 mL of 1% Trichloroacetic Acid (TCA) solution and centrifuged at 10,000×g for 10 min. The supernatant was added to 1 mL 0.5% (w/v) TBA made in 20% TCA. The mixture was heated in boiling water for 30 min and the reaction was stopped by placing the tubes in an ice bath. The samples were centrifuged at 10,000×g for 10 min and the absorbance of the supernatant was recorded at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value read at 600 nm. The level of lipid peroxidation was expressed as nmol/g fresh weight, with a molar extinction coefficient of 0.155/mM/cm.

The Hydrogen peroxide (H_2O_2): The Hydrogen peroxide (H_2O_2) contents in the leaves and roots were assayed according to the method of Velikova *et al*. (2000). Leaves and roots were homogenized in ice bath with 0.1% (w/v) TCA. The extract was centrifuged at 12,000×g for 15 min, after which to 0.5 mL of the supernatant was added 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI and the absorbance was read at 390 nm. The content of H_2O_2 was given on a standard curve.

Determination of total soluble protein content: Protein contents were assayed by following Bradford's method (Bradford, 1976) with BSA as standard. The absorbance was measured at 595 nm.

Antioxidant enzyme activity determinations: The leaves and roots of barley were collected for enzyme analysis. Fresh samples (1.0 g) were homogenized in ice-cold 50 mM phosphate buffer (pH 7.5). The homogenate was centrifuged at 12 000 g for 20 min at 4°C and the supernatants were used for the various enzymatic assays.

Catalase (CAT) activity was determinated according to Cakmak and Horst (1991). The reaction mixture for catalase in a total volume of 3 mL contained 50 mM Na-phosphate buffer (pH 7.2) and 300 mM H_2O_2. The reaction was started by adding enzyme extract and the activity was determined by monitoring the initial rate of H_2O_2 disappearance at 240 nm (ε = 39.4 /mM/cm).

Ascorbate Peroxidase (APX) activity was assayed according to the method of Nakano and Asada (1987). The reaction mixture consisted of 100 µL enzyme extract, 0, 5 mM ascorbate, 50 mM phosphate buffer (pH = 7, 2) and 100 µL H_2O_2 (300 mM). The oxidation of ascorbate was determined by the change in absorbance at 290 nm (ε = 2, 8 /mM/cm).

Guaiacol Peroxidase (POD) activity was measured according to the method of Putter (1974), with some modification. The reaction mixture (3 mL) consisted of 100 µL enzyme extract, 100 µL guaiacol (1.5%, v/v), 100 µL H_2O_2 (300 mM) and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). Any increase in the absorbance due to oxidation of guaiacol was measured spectrophotometrically at 470 nm (ε = 26.6 /mM/cm).

Statistical analysis: All values reported in this study are the mean of at least three replicates. For each parameter, data were subjected to a one-way ANOVA analysis. When the effect was significant ($p = 0.05$), differences between means were evaluated for significance by using Tukey's (HSD) test (MINITAB software version 16.0).

RESULTS

Chlorophyll and carotenoid contents: Table 1 shows the influence of $CdCl_2$ on the photosynthetic pigments chlorophyll (a, b, a+b) and carotenoids in the leaves of barley seedlings. The growth retardation of barley plants by cadmium application was found to be associated with a significant decrease in chlorophyll a, b and a+b content. A substantial decline in chlorophyll a and b was observed at 100 µM. Compared to control; total chlorophyll content showed approximately 45% and 55% reduction in 50 and 100 µM cadmium treated plants respectively. Cadmium addition suppressed chl (a, b, a+b) and carotenoids contents in all treatments.

Table 1: Changes of chlorophyll and carotenoid contents (mg/g FW) in leaves of barley seedlings induced by $CdCl_2$

Treatments (µM)	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl a+b (mg/g FW)	Carotenoids (mg/g FW)
Control	3.70±0.46a	7.16±1.13a	10.86±0.77a	2.20±0.38a
25	3.30±0.62ab	5.67±0.31a	8.97±0.85b	1.24±0.48ab
50	2.35±0.47bc	3.68±0.59b	6.06±0.22c	1.12±0.43b
75	2.01±0.44c	3.36±0.41b	5.28±0.82c	0.74±0.20b
100	1.56±0.22c	3.26±0.45b	4.93±0.57c	0.64±0.17b

Data are presented as mean±S.E. of three replicates for each parameter; The same letters after the data indicate that there is no significant difference at a probability level of 95% (Tukey's test)

Table 2: Effect of $CdCl_2$ on the Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) concentrations of barley plants

	MDA (nmol/ (g FW))		H_2O_2 (nmol/ (g FW))	
Treatments (µM)	Leaves	Roots	Leaves	Roots
Control	3.08±0.42c	1.04±0.58d	32.48±2.80b	43.68±2.11c
25	4.78±1.03bc	2.83±0.44cd	36.02±2.24b	49.37±3.98c
50	5.32±0.22bc	3.36±0.59c	46.57±3.08b	77.84±4.78b
75	6.14±1.23b	6.94±0.72b	68.78±4.24a	85.86±4.30b
100	10.09±1.76a	12.07±1.15a	81.10±12.30a	107.52±1.70a

Data are presented as mean±S.E. of three replicates for each parameter; The same letters after the data indicate that there is no significant difference at a probability level of 95% (Tukey's test)

Table 3: Effect of $CdCl_2$ on the total soluble protein content of barley plants

	Total soluble protein (mg/g FW)	
Treatments (µM)	Leaves	Roots
Control	2.79±0.11d	1.17±0.34e
25	3.41±0.34d	3.02±0.10d
50	7.33±0.64c	6.65±0.19c
75	10.13±0.48b	9.38±0.90b
100	12.16±0.34a	10.95±0.51a

Data are presented as mean±S.E. of three replicates for each parameter; The same letters after the data indicate that there is no significant difference at a probability level of 95% (Tukey's test)

The changes in chlorophylls contents were statistically highly significant ($p<0.001$). The concentrations of carotenoids in leaves decreased as the cadmium supply was increased significantly ($p<0.01$) from 2.20 mg/g FW in the control to 0.64 mg/g.

Lipid peroxidation (MDA) and Hydrogen peroxide (H_2O_2) contents: The MDA and H_2O_2 contents in both the roots and leaves of barley plants are shown in Table 2. The application of $CdCl_2$ (25, 50, 75 and 100 µM, respectively) of barley plants results in very significant ($p<0.001$) for the production of MDA in leaves and roots in all treated plants compared to the control. Treatment with cadmium in the roots compared to the control product a change in the synthesis of lipoperoxides. These increases relative to the control from the first concentration $CdCl_2$ and they multiply from 50 µM and strongly accumulate at the 100 µM concentration. The H_2O_2 content in both the roots and leaves of barley plants are shown in Table 2. The H_2O_2 content in the leaves and roots of barley plants increased markedly ($p<0.001$) when the plants were exposed to cadmium stress. The accumulation of H_2O_2 increased in leaves of barley at higher cadmium concentrations, H_2O_2 peaked at 100 µM in leaves. The content of H_2O_2 in roots increased and peaked at different concentrations of $CdCl_2$. Thus, we see that the increase of the synthesis of H_2O_2 is higher in roots than in the barley leaves.

Total soluble protein content: Table 3 shows the change in total soluble protein content in the roots and leaves of barley plants. From the results, we observe a very highly significant increase in total soluble protein in the roots and leaves of the treated plants compared to the control ($p<0.001$).

Antioxidant enzyme activity: The changes in antioxidative enzymatic activities in barley roots and leaves, including APX, POD and CAT, induced by Cadmium at different concentrations are shown in (Table 4).

Cadmium caused a marked induction in APX activity in leaves and roots of barley. APX was increased significantly in leaves and roots barley with 100 µM $CdCl_2$ treatment as compared to the control. The changes in APX activity were also statistically significant among the different concentrations of $CdCl_2$ ($p<0.001$).

Catalase is a key enzyme in protecting cells against oxidative stress. In the present work, CAT activities in leaves and roots barley were increased at all $CdCl_2$ treatments compared with the control (Table 4). The highest Catalase activity of the barley roots and leaves occurred at 100 µM $CdCl_2$. Furthermore, the changes in CAT activities were statistically significant among all treatments ($p<0.001$).

Among various enzymes involved in the elimination of ROS gaïacol peroxidase can be considered one of the key enzymes, since both of its extra and intracellular forms are participating in the breakdown of H_2O_2. POD in leaves and roots of barley plants increased markedly ($p<0.001$) when the plants were exposed to Cadmium stress. Compared to the controls, POD activity showed approximately 70% induction at 100 µM in roots and 81% increase at 100 µM of $CdCl_2$ treated leaves of barley.

Table 4: Effect of $CdCl_2$ on Catalase (CAT), Ascorbate Peroxidase (APX) and Peroxidase (POD) activities of barley plants

Treatments	CAT (nmol/mg protein/min)		APX (nmol/mg protein/min)		POD (nmol/mg protein/min)	
(µM)	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	22.80±2.59e	20.50±1.42e	46.79±8.04e	45.87±2.29d	41.99±2.06e	69.47±4.22e
25	45.04±2.80d	63.91±1.42d	68.40±8.43d	57.47±1.01c	127.17±2.38d	107.17±3.22d
50	64.41±2.28c	84.33±1.36c	104.94±2.42c	66.88±3.21b	150.69±1.41c	166.80±3.81c
75	73.10±1.95b	99.68±3.69b	139.05±2.36 b	78.59±3.37a	195.80±1.77b	201.69±6.43b
100	96.40±1.81a	123.39±1.54a	178.19±3.73a	85.17±4.49a	228.03±1.90a	226.47±2.11a

Data are presented as mean±S.E. of three replicates for each parameter; The same letters after the data indicate that there is no significant difference at a probability level of 95% (Tukey's test)

DISCUSSION

In plants, toxic metals induce oxidative stress by generating ROS viz. Hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), Hydroxyl radicals (OH^-) and singlet Oxygen ($^{\bullet}O_2$) (Devi and Prasad, 1998). ROS can rapidly attack and damage all types of bio-molecules including proteins, lipids and nucleic acids. On the other hand, plants can induce antioxidant enzymes, including POD, APX and CAT, to fight against oxidative damage under stress conditions (Ali *et al*., 2011).

Exposure of plants to toxic metals can cause many physiological and biochemical disorders. Concentrations of photosynthetic pigments are often measured to assess the impact of many environmental stresses. According to our results, Cadmium induces a lowering concentrations of chlorophyll (a, b, a+b) and carotenoids. This is consistent with numerous studies that report a reduction in the concentration of chlorophyll in the presence of cadmium (Wang and Zhou, 2006; Groppa *et al*., 2007; Belkhadi *et al*., 2010; Agami and Mohamed, 2013) and heavy metals in general (Mysliwa-Kurdziel and Strzalka, 2002; Lei *et al*., 2007). The decrease in chlorophyll is a primary event in plants subject to metal stress and results from the inhibition of the enzymes responsible for the biosynthesis of the chlorophyll (Mysliwa-Kurdziel and Strzalka, 2002). The stress induced by cadmium decreases the rate of assimilation of CO_2 causing disruption in the process of photosynthesis and the chlorophyll degradation and inhibition of its biosynthesis; which could lead to disruptions in the transport of electron flow PSI and PSII leading to the reduction of O_2 and the generation of ROS (Moussa, 2004).

Cadmium also induces a decrease in the concentration of carotenoids; this is consistent with many studies on various plants (Mysliwa-Kurdziel and Strzalka, 2002; Belkhadi *et al*., 2010). The high accumulation of cadmium in leaves of barley plants is probably responsible for the production of ROS; this can cause partial destruction of antioxidants such as carotenoids.

According to our results, we also note that the leaves and roots of plants exposed to cadmium have high levels of H_2O_2 and MDA as a result of the generation of ROS. The increase of MDA production indicates increased lipid peroxidation. It is well known that the peroxidation of polyunsaturated fatty acids of membrane phospholipids and causes a deficit of membrane functions, in particular through reduction of the fluidity and the inactivation of enzymes and receptors located at the membranes (Lagadic *et al*., 1997). This can then participate in a change in membrane permeability. It is also possible that partial exclusion of Cadmium results from a change of the capacity of the cell walls to bond to the metal or an increased excretion of chelating substances as reported by Ghosh and Sigh (2005) and Kirkham (2006).

As regards hydrogen peroxide, we have observed a significant increase of H_2O_2 in leaves and roots; this may be related to oxidative damage the membrane. Foyer *et al*. (1997) indicates that H_2O_2 is a strong oxidant that can initiate localized oxidative damage leading to disruption of function and loss of metabolic cell integrity at sites where it accumulates. H_2O_2 and other ROS may be responsible for lipid peroxidation. H_2O_2 can release at relatively long distances causing changes in the redox status of tissues and surrounding cells to which relatively low concentrations or triggers an antioxidant response. These results may indicate that the harmful impact of toxic metals on plants is probably exerted by ROS production.

Numerous studies have demonstrated an increase in total protein in plants under different stress (heavy metals, pesticides and drought) (Haiyan *et al*., 2005; Zhiqiang *et al*., 2009). We observed similar results in our work, especially in the roots of barley treated with cadmium. Moreover, the accumulation of total protein in the leaves and roots of barley may be related to oxidative damage generated by the toxicity of cadmium. Indeed, the variation of the levels of total protein is linked firstly to the variation of the enzymatic activities (Zhou *et al*., 2004). On the other hand, in response to the substantial ROS production plants develop antioxidant defense mechanisms including the induction of stress protein (Sanita di Toppi and Gabbrielli, 1999; Shah *et al*., 2001).

The accumulation of intracellular ROS in situations of environmental stress leads to the activation of defense mechanisms by increasing antioxidant enzyme activities or mechanisms to repair oxidative damage (Ramel *et al*., 2009). Indeed, according to our results

we found that cadmium tends to stimulate the synthesis of antioxidant enzymes (APX, POD and CAT) in leaves and roots of barley. Similar results have been reported by many studies (Hegedüs *et al*., 2001; Aravind and Prasad, 2003; Tiryakioglu *et al*., 2006; Touiserkani *et al*., 2012). Levels of APX, CAT and POD increased under certain environmental conditions such as the presence of high concentrations of salt or heavy metals (Sugimoto and Sakamoto, 1997). In response to increased ROS, the antioxidant defense system including POD, APX and CAT plays an important role in ROS scavenging (Sandalio *et al*., 2001; Ali *et al*., 2013; Issaad *et al*., 2013). However, according to our results we note that the defense system could not regulate the concentration of H_2O_2 produced by the stress induced by cadmium was strongly accumulated in leaves and roots of barley.

These results are consistent with previous reports by Chou *et al*. (2012) for rice and Mohamed *et al*. (2012) for Indian mustard, indicating that antioxidant enzymes are not a sufficient defense system. A drastic increase in H_2O_2 may consequently have a lower extensibility of plant cell walls, which can rapidly terminate growth (Shutzendubel and Polle, 2002).

CONCLUSION

The results related to the effects of the toxicity of cadmium on enzyme activities are very controversial. The reason for such inconsistent results on the effects of cadmium seems to be related to some differences in the study material:

- Plant organ studied (root, leaf)
- Duration of exposure and concentration of cadmium used
- Cultivars and genotypes (or plant species) considered in the studies (Tiryakioglu *et al*., 2006)

The results obtained in the present work showed that the barley Saida is a sensitive and non-tolerant cultivar to cadmium toxicity. The sensitivity of this cultivar is associated with increased enzyme activity and levels of hydrogen peroxide and registered trapping defense mechanisms. In perspective, in the future we should pay particular attention to the study of the impact of cadmium on different cultivars of barley to upgrade the culture of barley in Algeria.

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