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SHORT COMMUNICATION

ANTIOXIDATIVE DEFENSE TO LEAD STRESS IN ROOT CELLS OF *CICER ARIETINUM* L.

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The study was conducted to evaluate the lead (Pb) induced changes at the cellular levels in the *Cicer arietinum* L. cv. Abrodhi. The seedlings were grown hydroponically, and subjected to increasing concentration of Pb (0, 0.5, 1.0 and 2.0 mM) as $Pb(NO_3)_2$ for 5 days. The activities of enzymes involved in antioxidative defense such as superoxide dismutase (SOD), guaiacol peroxidase (POD) were markedly enhanced while catalase (CAT) decreased prominently with increasing the concentration of Pb. The non-enzymatic antioxidative scavengers, viz. ascorbic acid, non-protein thiols, phenol and proline contents showed a progressive increase during the time of Pb treatment. The maximum increase or decreases in the level of the above biochemicals were observed at 2.0 mM concentration. The results suggested that Pb toxicity causes oxidative stress in seedlings and the antioxidative enzymes play a pivotal role against oxidative injury.

***Key words*: Antioxidative defense, *Cicer arietinum*, lead, oxidative stress, superoxide dismutase**

Lead (Pb) is one of the major heavy metal of the antiquity and has gained considerable attention as a potent environmental pollutant. Apart from the natural weathering processes, Pb contamination of the environment has resulted from mining and smelting activities, Pb containing paints, gasoline and explosives as well as from the disposal of municipal sewage sledges rich in Pb (Chaney and Ryan 1994). Despite regulatory measures adopted in many countries to limit Pb input in the environment, it continues to be one of the most serious global environmental and human hazards. As many of the Pb pollutants are indispensable for modern human life, soil contamination with Pb is not likely to decrease in the near future (Yang *et al*. 2000).

Previous studies have shown that Pb inhibits metabolic processes such as nitrogen assimilation, photosynthesis, respiration, water uptake, and transcription (Krupa *et al*. 1993). Similarly as those of other heavy metals, lead ions (Pb^{2+}) can intensify the processes of reactive oxygen species (ROS) production leading to oxidative stress (Cuypers *et al*. 1999). It is well documented that increase in oxidative stress conditions lead to increased production of ROS (Foyer *et al*. 1997). These processes, which destructively effect cell structure and metabolism, are mutually connected and stimulate each other, which may result in a decreased efficiency of oxidation-reduction enzymes or the electron transport system leading to fast production of ROS in the cell (Stroinski and Kozlowska 1997). In extreme cases, when ROS level exceeds the capacity of cell defense mechanisms, structural and functional damage takes place, leading to cell death (Raha and Robinson 2000). The main sites of ROS formation in

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plant cells are chloroplasts, peroxisomes and mitochondria. Generally in plants, ROS are synthesized during the photosynthetic electron transport in chloroplast. Under stress conditions, like heavy metal stress, the photosynthetic electron can not be used in the reduction of CO_2; therefore, they accumulate in chloroplasts and are then transferred to molecular O_2. Because of O_2 activation, highly reactive oxygen species ($O_2^{\cdot-}$, superoxide radical; H_2O_2, hydrogen peroxide; OH^-, hydroxyl radicals and $^1O_2$, singlet oxygen) are produced (Asada 2000).

The antioxidative system of the cell developed different enzymatic and non-enzymatic defense mechanisms against oxidative stress induced by ROS. Among the enzymatic reactions, superoxide dismutase (SOD) is involved in detoxification of $O_2^{\cdot-}$, catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD) are involved in detoxification of H_2O_2 (Asada 1992). Antioxidants like ascorbic acid (vitamin C), ±-tocopherol (vitamin E), non-protein thiols (glutathione), carotenoids, phenols and proline are the most important non-enzymatic defense metabolites of plants against ROS.

Information on the relationship between heavy metal effects and oxidative stress in plants is rather scarce. In the present communication the possible antioxidative mechanisms that could be operational in the roots of *Cicer arietinum* seedlings exposed for 5 days to environmentally relevant (0.5 mM) as well as to marginally acute (2.0 mM) concentrations of Pb in hydroponic cultures were assessed.

Plant material and treatment: Seeds of *Cicer arietinum* L. cv. Abrodhi (chickpea) were sterilized with 1% NaClO for 5 min, then washed twice with distilled water and germinated for 4 days in the dark in petri dishes. Four-day old seedlings were grown in 1/4 strength modified Hoagland nutrient solution (Pickering *et al*. 2000). Seedlings were grown at 24^0C, with a light intensity of 300 µmol m^{-2} s^{-1} and a 14-h photoperiod. After growing for 10 days (four true leaves), they were treated with 0, 0.5, 1.0 and 2.0 mM Pb^{2+} as $Pb(NO_3)_2$ for 5 days. The pH was adjusted to 5.5 for both culture and treatment solution and renewed everyday. The roots of seedlings were collected and washed in 10 mM $CaCl_2$ to remove Pb accumulated on their surface and stored in freezer at -80^0C.

Dry weight determination: One gram fresh weight of root from various samples were taken, wrapped in aluminum foil and oven dried at 70^oC in hot air oven until a constant weight was recorded. *Assays of antioxidative enzymes*: Approximately 0.5 g fresh root samples were homogenized in 50 mM potassium phosphate buffer (pH 7.6) including 0.1 mM Na-EDTA. Samples were generally homogenized in 10 ml, and then centrifuged for 15 min at 20,000 rpm at 4^0C. Catalase and superoxide dismutalse activities were assayed according to the methods described by Cakmak and Marschner (1992). Guaiacol peroxidase activity was determined spectrophotometrically by the method of Putter (1974).

Determination of ascorbic acid: Ascorbic acid (AsA) content in the roots of seedlings was determined according to Cakmak and Marschner (1992) with some modification. 0.5 g root samples were extracted with 5.0 ml of 5 % meta-phosphoric acid, and centrifuged at 4000 rpm for 30 min. The reaction mixture contained 0.2 ml aliquot of the 4000 rpm supernatant, 0.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.1 ml 10 mM 1, 4-dithiothreitol (DTT) and 0.1 ml 0.5 % (w/v) N-ethylmaleimide (NEM) to remove excess DTT. In the reaction mixture, the color was developed after addition of the following reagents: 0.4 ml 10 % trichloroacetic acid (TCA), 0.4 ml 44 % ortho-phosphoric acid, 0.4 ml 4 % 2, 2'-bipyridine in 70 % ethanol, and 0.2 ml 3 % $FeCl_3$. The mixture was then incubated at 40^0C for 40 min, and the absorbance was measured at 525 nm.

Determination of non-protein thiols: Non-protein thiols (NPT) were extracted by grinding 0.5 g roots in 1.0 ml ice-cold 5% (w / v) sulfosalicylic acid solution. After centrifugation at 10 000 rpm at 4ÚC for 30 min, the supernatants were collected and immediately assayed. NPT was measured with Ellman's reagent (Ellman 1959). Briefly, 300 ¼L of the supernatant was mixed with 1.2 mL of 0.1 M phosphate buffer solution (pH 7.6). After a stable absorbance reading of 412 nm was

obtained, 25 ¼M 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution (6 mM DTNB dissolved in 5 mM EDTA, 0.1 M PBS, pH 7.6) was added, and the increase in absorbance at 412 nm was monitored.

Determination of phenol and proline: Phenols were extracted according to the method given by Bray and Thorpe (1954) and proline concentration in roots of seedlings was determined by the method of Bates *et al*. (1978). The data recorded during the course of investigations were subjected to statistical analysis "Analysis of variance" technique for drawing conclusions. The significant and non-significant treatment effect was judged with a help of F table.

Though, heavy metals are integrated component of the ecosystem, uptake of higher concentration of heavy metal is found to be toxic for plants. A decrease in root dry mass and CAT activity with a simultaneous increase in POD and SOD activities in seedlings treated with Pb was observed (Table 1). The progressive increasing trend was also observed in all the non-enzymatic scavengers analyzed in our experiment, *viz.* AsA, NPT, phenol and proline under Pb treatment (Table 2).

Table 1 showed that treatment of seedlings with various levels of Pb significantly ($p<0.05$) reduced the root dry mass by 22.85, 50.46 and 70.71% respectively against control. The lowest value recorded was in *Cicer arietinum* roots treated with 2.0 mM Pb with the value of 0.653 g plant^{-1} (Table 1).The reduction in the specific activity of CAT was observed in roots of seedlings treated with different concentrations of Pb, the lowest value recorded was 192.00 unit mg^{-1} protein (Table 1) and the significant ($p<0.05$) percent decrement of 10.23, 21.38 and 33.56 was observed at 0.5, 1.0 and 2.0 mM Pb supply respectively as compared to control (Fig. 1 B). Activities of POD displayed a progressive increase in response to Pb in a dose-response experiment, and the peak activity 387.40 units mg^{-1} protein was found at 2.0 mM Pb concentration (Table 1). Specific activity of POD in roots was increased significantly ($p<0.05$) with 11.48, 28.37 and 29.36% respectively at 0.5, 1.0 and 2.0 mM Pb treatment as compared to control (Fig. 1 C). SOD activity significantly increased at all concentrations over control. The maximum value recorded was 275.64 unit mg^{-1} protein at 2.0 mM Pb treatment (Table 1) and increment of 4.0, 21.06 and 53.04 % were recorded at 0.5, 1.0 and 2.0 mM Pb supply as compared with control (Fig. 1 D).

Upon exposure to increasing the dose of Pb from 0.5 to 2.0 mM, the significant increment ($p<0.05$) of AsA with the value 28.29, 49.57 and 67.37% were recorded against control (Fig. 2 A) and the highest mean value was found 0.753 mg 100g^{-1} fw at 2.0 mM Pb concentration (Table 2). With increasing supply of Pb, the content of NPT were increased significantly ($p<0.05$) 14.49, 46.95 and 74.31% compared to control (Fig. 2 B). The maximum value was observed 94.26 nmol g^{-1} fw at

Table 1. Dry mass and activity of catalase, guaiacol peroxidase and superoxide dismutase in *Cicer arietinum* roots.

Treatments	Lead concentration (mM)	Dry mass (g plant^{-1})	Catalase (units mg^{-1} protein)	Guaiacol peroxidase (units mg^{-1} protein)	Superoxide dismutase (units mg^{-1} protein)
Control	0	0.653 ± 0.02	386.00 ± 9.27	211.53 ± 7.18	84.56 ± 2.70
Pb	0.5	0.410 ± 0.02 (37.22%)	314.33 ± 7.40 (18.57%)	266.44 ± 10.94 (25.9%)	91.61 ± 2.57 (8.33%)
	1.0	0.215 ± 0.01 (67.08%)	250.00 ± 6.68 (35.24%)	319.77 ± 3.93 (51.17%)	129.68 ± 3.54 (53.35%)
	2.0	0.112 ± 0.01 (82.85%)	192.00 ± 7.39 (50.26%)	387.40 ± 5.97 (83.14%)	275.64 ± 9.69 (225.96%)
CD		0.21	21.46	18.96	14.63

In parenthesis indicate % increase or decrease over control

2.0 mM Pb concentration (Table 2). Phenol contents were high in the entire seedlings grown in nutrient solution under Pb treatments (Table 2). The significant increment ($p<0.05$) of phenol content in the roots of seedlings were recorded 31.00, 47.85 and 53.07% respectively at 0.5, 1.0 and 2.0 mM Pb against control (Fig. 2 C). The proline contents were monitored high in the roots of seedlings grown under Pb supply hydroponic culture medium (Table 2). The significant ($p<0.05$) increments of proline were recorded 5.12, 22.79 and 61.42% over control depicted in Fig. 2 D.

In the present study, several biochemical and physiological responses representing the oxidative damage and protection in roots of *Cicer arietinum* seedlings were examined. Pb moves predominantly into the root apoplast and thereby in a radial manner across the cortex and accumulates near the endodermis. The endodermis acts as a partial barrier to the movement of Pb between the root and shoot. This may in part account for the reports of higher accumulation of Pb in roots compared to shoots (Verma and Dubey 2003). The adverse effect of excess Pb in the roots probably caused the decrease in dry mass content (Jarvis and Leung 2002). The rapid uptake of heavy metal was observed in *Brassica juncea* L. immediately after the start of treatment and the 50% reduction in root dry mass was observed (Wang *et al*. 2004). Our experiment results showed that the amount of Pb in the roots increased with greater rates and the dry mass decrease. CAT is an indispensable enzyme required for ROS detoxification, located in peroxisomes participate in the breakdown of the photorespiratory H_2O_2 to H_2O and molecular oxygen or peroxidatively of H donors in the presence of H_2O_2 (Foyer 1995). Decreased activity of the CAT in the roots of *Cicer arietinum* seedlings indicates that it might be due to increase in H_2O_2 that in turn inactivates or suppress the enzyme under stress condition of Pb treatment. Panda and Patra (1988) have also reported a decrease in CAT activity in response to Zn in rice, wheat and green gram.

The stimulation of peroxidase capacity in seedlings indicates increased level of H_2O_2 in root tissues, generated after exposure of seedlings to Pb and an attempt of plant to protect them against oxidative stress. Schutzendubel *et al*. (2001) reported that treatment of heavy metal at higher concentration induced increases in POD activities which was accompanied by accumulation of phenolics and lignification that might be due to consumption of H_2O_2, lead to decrease in Pb-induced oxidative stress in plant. The increased activity of POD in seedlings may also be an indication of enhanced senescence caused by heavy metals (Sandalio *et al*. 2001).

Increased SOD activity in the root tissues of the plants is due to the generation of active oxygen species under Pb toxicity. Scandalios (1997) explained obtained result assuming that oxidative stress could cause increased turnover and re-synthesis of SOD with no net change in its concentration. Within a cell, SOD constitutes the first line of defense against ROS. $O_2^{\cdot -}$ is produced at any location where an electron transport chain is present and hence oxygen activation may occur in different compartments of the cell (Elstner 1991). The

Table 2. Content of ascorbic acid, non-protein thiols, phenol and proline in *Cicer arietinum* roots.

Treatments	Lead concentration (mM)	Ascorbic acid (mg 100 g^{-1} plant)	Non-protein thiols (nmol g^{-1} fw)	Phenol (mg 100g^{-1} fw)	proline (µmol g^{-1} fw)
Control	0	0.147 ± 0.04	13.89 ± 1.44	0.316 ± 0.01	0.293 ± 0.02
Pb	0.5	0.263 ± 0.02 (78.91%)	18.60 ± 1.89 (33.90%)	0.600 ± 0.02 (89.87%)	0.325 ± 0.01 (10.92%)
	1.0	0.436 ± 0.03 (196.59%)	38.48 ± 2.90 (177.03%)	0.896 ± 0.02 (183.54%)	0.466 ± 0.02 (59.04%)
	2.0	0.753 ± 0.03 (412.24%)	94.26 ± 4.42 (578.61%)	1.046 ± 0.05 (231.01%)	1.226 ± 0.13 (318.43%)
CD		0.019	3.47	0.068	0.137

In parenthesis indicate % increase or decrease over control

phospholipids membranes are impermeable to charged $O_2^{\cdot-}$, so therefore, it is crucial that SOD are present for the removal of oxygen in the compartments where $O_2^{\cdot}$ radicals are formed (Takahashi and Asada 1983). The SOD activity of a plant is increased by the use of high concentration of heavy metal ions, by an increase in SO_2 concentration (Cutler *et al*. 1980).

Ascorbic acid (AsA) is a key antioxidant, and involved in protection of plant cells against oxidative damage catalyzed by ROS. Foyer and Harbinson (1994) reported that high amount of AsA product in plant cells in response to high concentration of heavy metal ions and detoxify the active oxygen species within the ascorbate-glutathione cycle. Ascorbic acid has an ability to scavenge a wide range of ROS: $O_2^{\cdot-}$, $^1O_2$, H_2O_2 and act as a chain breaking antioxidant. However, in the presence of the metal ions it acts as pro-oxidant (Beyer 1994).

The enhancement in NPT level might be considered as an indication for Pb to promote H_2O_2 production in chloroplast, and thus, activation of H_2O_2-scavenging ascorbate-glutathione cycle. NPT is also required for synthesis of metal-binding peptide such as phytochelatins (PCs), which bind and sequester metal in stable complexes in vacuoles (Clemens 2001).

It is suggested that the polymerization of polyphenols by peroxidases, enhanced after Pb uptake and detoxification (Backman *et al*. 1972). Moreover it has been shown that phenolic compound can be involved in the H_2O_2 scavenging cascade in the plant cells (Takahama and Oniki 1997). Our above given findings were correlated with the Anthony *et al*. (1982), who reported the concentration of total phenolic compounds in hydroponically grown *Helianthus annus* L.

In many plants under various forms of heavy metal stress, the concentration of proline increases up to 80 % of the amino acid pool. In addition to its role as an osmolyte and a reservoir of carbon and nitrogen, etc proline has been shown to protect plants against free radical-induced damage (Alia *et al*. 2000). The possible role of proline under metal stress have been proposed with greater or lesser convictions, which include stabilization of protein, scavenging of hydroxyl radicals, quenching of singlet oxygen, regulation of the cytosolic pH and regulation of NAD/NADH ratio (Matysik *et al*. 2002) .

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