ROLE OF SALICYLIC ACID IN REGULATING CADMIUM INDUCED OXIDATIVE STRESS IN *ORYZA SATIVA* L. ROOTS

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Summary. In the present investigation we studied the role of salicylic acid in cadmium induced physiological and biochemical changes and the possible induction of oxidative stress in developing rice roots. 24h cadmium treatment resulted in the inhibition of root dry biomass, root elongation and increased cadmium accumulation in roots. Salicylic acid presoaking for 16hrs resulted in protection against cadmium, minor changes in root dry biomass, root elongation and minimal accumulation of cadmium. Cadmium applied to salicylic acid free plants increased the level of lipid peroxidation, hydrogen peroxide content and superoxide radical production. Salicylic acid treatment decreased the toxic level of cadmium manifested by lower lipid peroxidation, lesser production of hydrogen peroxide and reduction in the generation of superoxide radical. The extent of membrane integrity loss was higher in salicylic acid free plants compared to salicylic acid treated ones, under cadmium application. To test the antioxidant efficiency of various enzymes, the activity of superoxide dismutase, catalase, peroxidase and glutathione reductase were measured. Higher increase in the activity of these antioxidant enzymes was observed in salicylic acid free plants than in the salicylic treated ones. Elevated level of ascorbate and glutathione was observed in salicylic acid free plants. Glutathione, however, decreased at higher concentration. Data show the potentiating effect of salicylic acid in regulating cadmium induced oxidative stress in *Oryza sativa* L. roots.

Keywords: antioxidant, cadmium, oxidative stress, lipid peroxidation, *Oryza sativa*, salicylic acid

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Abbreviations: BHT – butylated hydroxyl toluene, CAT – catalase, Cd – cadmium, DW – dry weight, FW – fresh weight, GPx – guaiacol peroxidase, GR – glutathione reductase, LMI – loss of membrane integrity, LPx – lipid peroxidation, MDA – malondialdehyde, ROS – reactive oxygen species, SA – salicylic acid, SOD – superoxide dismutase, TBA – thiobarbituric acid, TCA – trichloroacetic acid

INTRODUCTION

Cadmium (Cd) is a strong environmental pollutant with high toxicity to animals and plants. It is released from metals, phosphate fertilizers (Nriagu and Payma, 1988) and rock mineralization process (Baker et al., 1990). The high mobility of this metal in soil-plant system makes easier its entrance to the food chain (Nogawa et al., 1987). Cd penetrates the root via cortical tissues and reaches the xylem by apoplastic or symplastic pathways (Salt et al., 1995) where it forms complexes with several ligands, organic acids and phytochelatins (Caltado et al., 1988; Prezemeck and Hasse, 1991; Senden et al., 1992; Sanden et al., 1994). Cd toxicity in plants causes leaf rolls, chlorosis and reduced growth of both root and stem. It interacts with the water balance and damages the photosynthetic apparatus (Krupa, 1988; Barceló and Poschenrieder, 1990; Costa and Morel, 1994). Cd alters the level of enzyme activities of metabolic importance, disturbing the normal physiological process in plants (Mattioni et al., 1997). It can increase the permeability of membrane and inhibit the activity of membrane bound ATPase.

Cd induces oxidative stress in plants (Hendry et al., 1992; Somashekaraiah et al., 1992). Unlike other heavy metals such as copper (Cu), Cd cannot generate reactive oxygen species (ROS) via Haber-Weiss or Fenton type reactions (Salin, 1988). Cd toxicity results in the alteration of oxidant level in plants, inducing the generation of toxic reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), superoxide radical (O_2^-), etc. (Gallego et al., 1996; Choui et al., 1997). ROS generated in cells are highly reactive in nature and destroy the normal cellular function and metabolism. Plants have various defense mechanisms by which they can scavenge these ROS. Cd enhances the activity of some antioxidant enzymes like catalase, peroxidases, superoxide dismutase and glutathione reductase and non-enzymes like ascorbate, glutathione, α-tocopherol (Panda, 2002). The varying response to Cd-induced oxidative stress can be related to the amount of Cd supplied and also the concentration of thiol groups that are either present or induced by Cd. Thiols possess efficient antioxidative properties that can counteract oxidative stress (Pichorner et al., 1993).

Salicylic acid (SA) is an important signal element and endogenous growth regulator involved in local and endemic disease resistance in plants (Enyedi et

al., 1992; Alvarez, 2000). Earlier investigations have shown the role of SA in modulating plant responses to a wide range of oxidative stresses (Shirasu et al., 1997). Exogenous application of SA induces thermotolerance in mustard and maize by increasing the antioxidant efficiency, which in turn induces chilling tolerance (Raskin et al., 1987; Dat et al., 1998; Janda et al., 1999). Responses of SA to Cd has been observed in barley where SA applied for 24hrs resulted in Cd tolerance (Matewally et al., 2003). An increase in wheat salinity resistance under SA treatment, was reported by Shakirova and Bezrukova (1997). SA induces resistance to water deficit and ameliorates the damaging effects of heavy metals, like lead and mercury (Mishra and Choudhuri, 1999; Bezrukova et al., 2001). One of the important roles of SA in inducing resistance to various environmental stresses is manifested by its ability to express genes that code for PR-proteins (Merkouropoulos et. al., 1999).

In the present investigation we report the effects of SA on Cd induced oxidative stress and antioxidative metabolism in *Oryza sativa* L. roots.

MATERIALS AND METHODS

Plant material and experimental design

Dry graded seeds of *Oryza sativa* L. (cv: Longai) were collected from Regional Agricultural Research Station, Karimganj. Seeds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) and washed thrice with deionized water. Sterile seeds were germinated in Whatman No.1 filter paper for 3d at $30^{\pm}2^0$C. For SA treatment, seeds were soaked in 100 µM SA for a period of 16 hrs and then germinated as described above. Germinated seeds were transferred to plastic cups supplied with balanced Yoshida nutrient solution. The plants were kept inside growth chamber under continuous white light (*Phillips, 20W TLD,* India) with photon flux density of 52 µmoles m^{-2} s^{-1} (PAR) and 16 hrs photoperiod. After 3 days of growth, Cd was applied in the form of cadmium chloride ($CdCl_2$) at different concentrations (0, 10, 100 and 1000 µM). After 24 hrs, the roots were harvested for analysis.

Growth and Cd accumulation

The growth of the root was studied in terms of root length and dry biomass. Root length was measured using a centimeter scale. For dry biomass measurement, roots were dried at 70ºC for 2 days and weighed. The dried roots were digested in glass tubes containing 5ml concentrated nitric acid (HNO_3) at 100ºC until the solution turned clear. The final volume was adjusted to 20 ml with distilled water. Total Cd content was measured using atomic absorption spectrometer (Perkin-Elmer, 3110).

Quantification of lipid peroxidation and loss of membrane integrity

Lipid peroxidation was measured biochemically in terms of malondialdehyde (MDA), a peroxidation product of fatty acid from membrane lipids, according to Heath and Packer (1968). 0.2g of the root tissue was homogenized in 0.1% (m/v) cold trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000g for 20 min. To 1 ml supernatant root extract, 1 ml 20% TCA containing 0.5% thiobarbituric acid (TBA) and 0.01 ml butylated hydroxyl toluene (BHT, 4% solution in ethanol) were added. The mixture was then heated in water bath at 95^{o}C for 30 min, then cooled in ice. The samples were centrifuged at 10,000g for 15min and absorbance was recorded at 532 nm and corrected for 600 nm. MDA level was expressed using extinction coefficient 155 mM cm^{-1}. For histochemical studies, roots (tips) were stained with Schiff's reagent for 20 min. After the reaction, roots were rinsed in sulfite solution (O.5% potassium metabisulphite in 0.05N HCl). Stained roots were then mounted in glass slides and immediately photographed. Root loss of membrane integrity (LMI) was assessed according to Yamamoto et al. (2001). Roots were stained with Evans blue solution (0.025g Evans blue in 100 ml of 100 µM of calcium chloride, pH-5.6). Stained roots were washed with 200 ml of 100 µM calcium chloride after which the dye no longer illuted from the roots. 10 mm root sections were homogenized in 1% SDS by a microhomogeniser at room temperature and centrifuged at 15,000g for 10 min. The absorbance was recorded at 600 nm. Few stained roots were mounted in glass slide for histochemical analysis.

Hydrogen peroxide estimation

Total peroxide (H_2O_2) content was measured according to Sagisaka (1979). 0.2g of the root tissue was homogenized in 5% TCA and the homogenate was centrifuged at 17,000g at 0^{o}C for 10 min. The reaction mixture contained 1.6 ml of the supernatant root extract, 0.4 ml TCA (50%), 0.4 ml ferrous ammonium sulfate and 0.2 ml potassium thioccyanate. The absorbance was then recorded at 480 nm.

Enzymic and non-enzymic antioxidants determination

Antioxidant enzymes were extracted by homogenizing roots in 0.1M sodium phosphate buffer (pH-6.8) in pre chilled mortar pestle under cold conditions. The extract was centrifuged at 14,000g in a cooling centrifuge at 4^{o}C for 15 min. Supernatant was used for the assay of catalase (CAT) [EC 1. 11. 1. 6], guaiacol peroxidase (GPx) [EC 1. 11. 1. 7], glutathione reductase (GR) [EC 1. 6. 4 .2] and superoxide dismutase (SOD) [EC 1. 15. 1. 1]. CAT and GPX activities were measured according to Chance and Maehly (1955). The assay mixture was composed of 3 ml phos-

phate buffer (pH-6.8), 1 ml 30 mM H_2O_2 and 1 ml of enzyme extract. The reaction was stopped by adding 10% H_2SO_4 after 1 min incubation at 20°C. The acidified reaction was then titrated against 0.01M $KMNO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The CAT activity was expressed as µmol (H_2O_2 destroyed) g^{-1} (f.m.) min^{-1}. The 3 ml reaction mixture of GPX consisted of 0.1M phosphate buffer (pH 6.8), guaiacol (30mM), H_2O_2 (30mM) and 0.3 ml enzyme extract. The absorbance rate of change was measured at 420 nm. The GPx activity was expressed as µmol (H_2O_2 destroyed) g^{-1} (f.m.) min^{-1}. The assay of SOD was done according to Giannopoltis and Ries (1977). The 3 ml assay mixture consisted of 79.2 mM EDTA, 10.8 mM tetraethylene diamine, 0.0033% bovine serine albumin, 6 mM nitroblue tetrazolium (NBT), 600 µM riboflavin in 5 mM KOH and 0.2 ml enzyme extract. The reaction was initiated by placing the glass tubes in between fluorescent bulbs (*Philips 20W*). By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazen formation was read at 560 nm. Under the described above conditions, the increase in the absorbance (in the absence of enzyme) was 100%, and 50% initial was taken as an equivalent to 1 unit of SOD activity. GR was estimated according to Smith *et al.* (1988). The reaction mixture contained 0.2M phosphate buffer (pH 7.5) with 1 mM EDTA, 3 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.01M potassium phosphate buffer (pH 7.5), 2 mM NADPH, 1ml enzyme extract and distilled water to reach the final volume of 2.9 ml. Reaction was initiated by adding 2 mM oxidized glutathione or glutathione disulphide (GSSG). The increase in the absorbance was measured at 412 nm at 25°C over a period of 5 min. The activity was expressed as ΔA_{412} g^{-1} (f.m.) s^{-1}.

Non-enzymic antioxidants, ascorbate and glutathione were estimated according to Oser (1979) and Griffith (1980), respectively. Leaf tissues were homogenized in 5% (w/v) sulfosalicylic acid and then were centrifuged at 10,000g for 10 min. The reaction mixture for ascorbate consisted of 2 ml 2% Na – molybdate, 2ml 0.15N H_2SO_4, 1ml 1.5mM Na_2HPO_4 and 1 ml tissue extract. It was incubated at 60°C in water bath for 40min, cooled and centrifuged at 3000g for 10 min, and the absorbance was measured at 660nm. For glutathione estimation, tissue extract was neutralized in 0.5ml of 0.5 M potassium phosphate buffer (pH 7.5). The reaction mixture contained 0.5ml of 0.1 M Na – phosphate buffer (pH 7.5) with EDTA, 0.2 ml of 6 mM DTNB, 0.1ml of 2 mM NADPH and 1ml of 1–U yeast – GR Type III (*Sigma*, USA). The change in absorbance at 412 nm was followed at 25°C until it reached 5U.

Statistical analysis

All the experiments were repeated at least three times and data presented are means of three separate experiments, ± SE.

RESULTS

In the present experiment, we have examined the effect of Cd on developing rice roots after 24h treatment of hydrophonic culture, and the subsequent effect of SA in conferring tolerance to Cd in roots. 24h Cd appliance resulted in growth inhibition of the roots. Significant decline in root length and dry weight was observed in SA free roots. SA priming resulted in initial increase in root length and dry weight, with minor reduction at 1000 µM of Cd (Fig. 1, A, B). In the controls, no Cd was detected. Cd accumulation was significant in SA free roots as compared to the SA primed ones (Fig. 1, C).

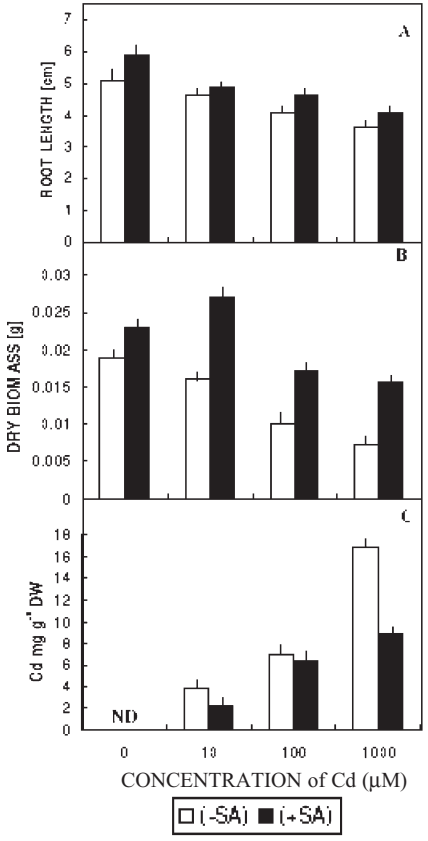

Fig. 1. Growth parameters: root length (A), root dry biomass (B) and bioaccumulation of Cd (C) in *Oryza sativa* L. under Cd toxicity and SA free/SA primed conditions. Data presented are the means of three separate experiments, ± SE.

Cd significantly affected the MDA production indicating lipid peroxidation. Increased MDA content was observed in roots at all three concentrations. In SA free roots, the increase was about 673% and 751.1% at 100 µM and 1000 µM, respec-

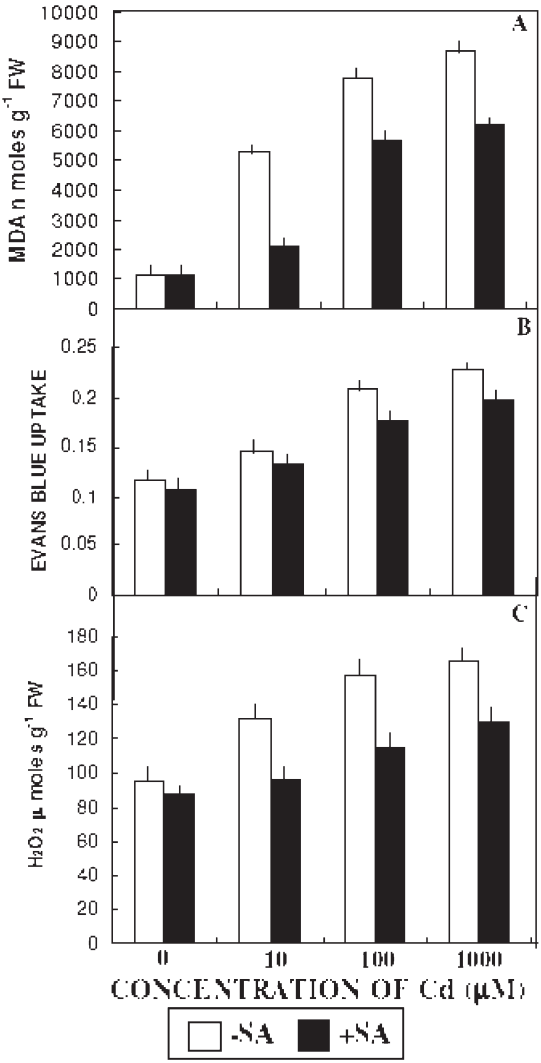

Fig. 2. MDA content (A), increase in membrane integrity loss (B) and total peroxide (H_2O_2) content (C) in SA free and SA primed roots of *Oryza sativa* L. under Cd toxicity. Data presented are the means of three separate experiments, ± SE.

tively (Fig. 2, A). Under SA primed conditions, the increase was significantly lower as compared to SA free controls, which showed 506.3% and 550.2% increases at 100 µM and 1000 µM Cd, respectively. Loss of membrane integrity, which signifies the membrane deterioration, was determined by measuring the Evans blue reagent uptake. Higher uptake was recorded in SA free plants as compared to the SA primed ones where the uptake was increased by 177.8% and 194.9% in SA free plants as

compared to 165.4% and 185% in SA primed ones at 100 µM and 1000 µM of Cd (Fig. 2, B). Lipid peroxidation and LMI were also detected histochemically. Patterns of the roots stained by Schiff's reagent and enhanced uptake of Schiff's reagent were observed at 100 µM and 1000 µM of Cd. Intense staining was observed in SA free roots. The enhancement of the Evans blue uptake was visible in roots by staining pattern as substantiated by the quantitative determination. Higher uptake of Evans blue was observed in roots under SA free conditions than that of SA primed ones (data not shown). Changes in total peroxide (H_2O_2) content are shown in Fig. 2 (C). An increase in the total peroxide content was observed in roots under Cd treatment, under SA free and SA primed conditions. Under SA free conditions, the H_2O_2 content increased by 138.5 %, 165.6 % and 173.8 % at 10 µM, 100 µM and 1000 µM concentrations which under SA primed conditions are about 110.6 %, 132 % and 148.4 % at these concentrations.

Changes in enzymic antioxidants are shown in Fig. 4(A–D). The CAT activity (Fig.4, A) showed an enhancement under Cd treatment in SA free plants. Minimum enhancement was observed in roots under SA primed conditions. Upon Cd treatment, the GPx activity (Fig. 4, B) showed an increasing trend in SA free plants. The increase in activity was higher at elevated concentration of Cd. Minimal increase was observed in SA primed plants. The activity of GR (Fig. 4,C) showed a similar trend under Cd treatment. SOD activity (Fig.4,D) showed initial increase at 10 µM and 100 µM Cd under SA free conditions. The activity increased by 175.9% and 251.7% at 10 µM and 100 µM concentration of Cd but decreased by 63.9% at 1000 µM in SA free plants. SA priming showed uniform increase in SOD activity which is lower than that of SA free plants. The activity increased by 146.3%, 238.2% at 10 µM, 100 µM and decreased at 1000 µM of Cd.

Non–enzymic antioxidants ascorbate and glutathione showed an increase in activity upon Cd treatment [Fig. 5(A–B)]. Ascorbate showed an increasing trend upon Cd treatment as compared to control under both conditions. The increase was highly pronounced at 100 µM and 1000 µM of Cd. At 10 µM, no significant change in ascorbate content was observed (Fig. 5,A). Glutathione showed initial increase at 10 µM under SA free and SA primed conditions, but gradually decreased at 100 µM and 1000 µM of Cd (Fig. 5,B).

DISCUSSION

In the present research, we have studied the effect of SA in regulating Cd induced oxidative stress in *Oryza sativa* L. roots. SA priming for 16hrs resulted in initial increase in growth and dry weight of roots, while in SA free roots Cd resulted in growth inhibition. Cd is known to inhibit plant growth (Aidid and Okamoto, 1993;

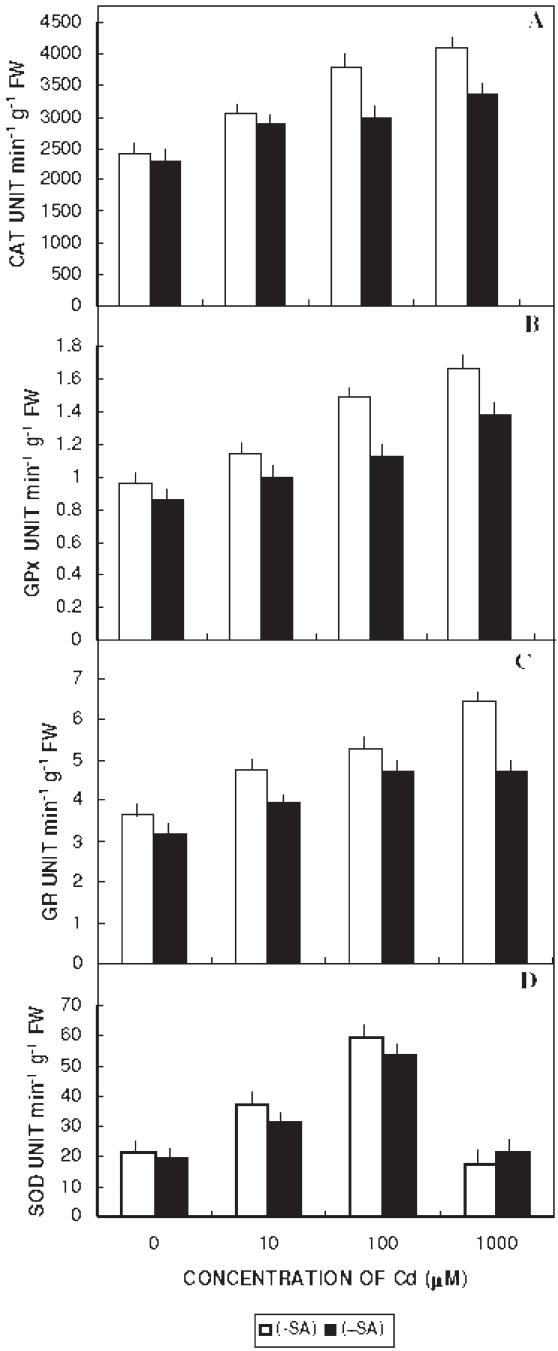

Fig. 4. Changes in the activity of antioxidant enzymes CAT (A), GPx (B), GR (C) and SOD (D) in SA free and SA primed roots of *Oryza sativa* L. under Cd toxicity. Data presented are the means of three separate experiments, ± SE.

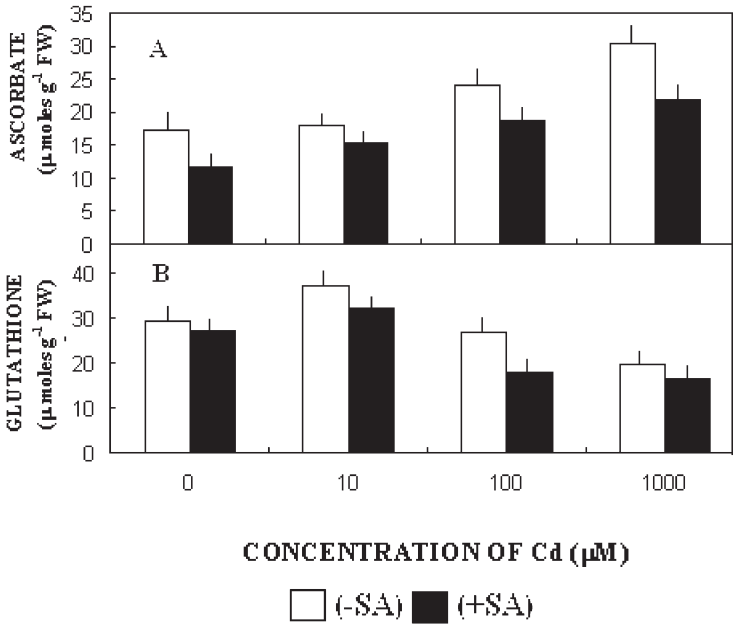

Fig. 5. Changes in non-enzymic antioxidant ascorbate (A) and glutathione (B) in SA free and SA primed roots of *Oryza sativa* L. under Cd toxicity. Data presented are the means of three separate experiments ± SE.

Veselov et al., 2003). SA growth inducing properties are reported in barley roots under Cd treatment (Matewally et al., 2003). Cd growth inhibition could be due to the inhibition of cell division and elongation rate of cells that mainly occurs by an irreversible inhibition of proton pump responsible for the process (Aidid and Okamoto, 1993; Liu et al., 2003/4). Usually, Cd is retained in roots and very small amount of it is transported in shoots (Caltado et al., 1983). Cd content in SA free roots was higher as compared to the one in SA primed roots. This differential accumulation of Cd can be considered as potentiating physiological effect of SA. In *Cassia tora*, SA treatment at different concentrations reduces the aluminum (Al) accumulation (Yang et al., 2003).

Oxidative stress is a response that results from increased levels of ROS in cells exposed to heavy metals (Dietz et al., 1999). H_2O_2 production rates increased in SA free roots upon Cd treatment. An increase in H_2O_2 production is reported in plants under various heavy metal treatments (Panda et al., a, b; Panda, 2003; Choudhury and Panda, 2004). As a consequence of ROS, deleterious to cells lipid peroxides are formed (Dietz et al., 1999; Panda, 2002). Under Cd treatment, an increase in MDA content indicated the oxidative stress in roots. MDA content, however, was lower in

roots pretreated with SA. The enhancement of MDA production and subsequent lowering under SA treatment was further substantiated by the histochemical staining pattern of the roots. As peroxidation products, aldehydes were produced. In SA free roots, staining was more intense. Similar results were obtained in pea roots treated with Al, and in *Lemna minor* under NaCl stress (Yamamoto et al., 2001; Panda and Upadhyay, 2004). Cd treatment resulted in an increase of membrane integrity loss (LMI). This increase was high in SA free roots. Such an increase is reported in pea under Al stress and *Lemna minor* under NaCl treatment (Yamamoto et al., 2001; Panda and Upadhyay, 2004).

In order to repair the damage initiated by ROS, plants evolve complex antioxidant metabolism. This includes enzymes like CAT, GPx, GR and SOD, and non-enzymes like ascorbate and glutathione. In SA free roots, Cd treatment resulted in the activation of CAT, GPx and GR. CAT and GPx are important enzymes that scavenge H_2O_2 detoxification in cells. Such results were reported in plants exposed to heavy metals like Cd, Cu and Pb (Van Assche and Clijsters, 1990; De Vos and Schat, 1991; Karataglis et al., 1991; Parta and Panda, 1998). SOD activity increase was observed in both SA free and SA primed roots. However, gradual decline was observed at 1000 μM of Cd. Such trend was reported in *Brassica juncea* under zinc (Zn) toxicity (Prasad et al., 1999). The change pattern of antioxidants in the presence of Cd under SA primed and SA free conditions, were reported in barley (Matewally et al., 2003).

Non-enzymic antioxidants, ascorbate and glutathione showed differential response. An increase in ascorbate content was observed in roots of both SA free and SA primed plants. Initial increase in glutathione was observed in roots of both SA and SA free plants with simultaneous decline at 1000 μM of Cd. Similar trend of non-enzymic antioxidants is observed in plants treated with Cr, Zn, Cu, Pb and As (Panda et al., 2003 a, b; Panda, 2003; Choudhury and Panda, 2004).

Roots suffer more injuries than shoots. In the present research, SA ameliorated Cd induced deleterious effects in roots. The increase in MDA production indicated oxidative stress that might be initiated by ROS under heavy metal stress. Cd induced oxidative stress was lower under SA treatment. This effect of SA acid can also be correlated to the changes in the antioxidant metabolism of roots. SA could form a complex with Cd that may also provide Cd tolerance. This effect was highly manifested after 16hrs SA pretreatment prior to germination. Antioxidant enzymes increased activities could also attributed to the increased tolerance to Cd. Our results suggest that SA can regulate Cd toxicity by activating Cd tolerance and regulating the antioxidant defense mechanisms.

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