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Cadmium-induced oxidative damage in mustard [*Brassica juncea* (L.) Czern. & Coss.] plants can be alleviated by salicylic acid

P. Ahmad [a,*], G. Nabi [b], M. Ashraf [c]

[a] *Department of Botany, Baramulla College, 193101, University of Kashmir, Srinagar, India*
[b] *Department of Biotechnology, Hamdard University, New Delhi 110062, India*
[c] *Department of Botany, University of Agriculture, Faisalabad, Pakistan; Second affiliation: King Saud University, Riyadh, Saudi Arabia*

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Abstract

The present study was carried out to examine the effects of salicylic acid (SA) on growth, activities of antioxidant enzymes and some physio-biochemical attributes in mustard [*Brassica juncea* (L.) Czern. & Coss.] plants exposed to cadmium (Cd) stress. Increasing concentrations of Cd led to decreased growth, shoot biomass, relative water content (RWC) and rate of photosynthesis (*A*). SA allayed the adverse effects of Cd on growth, RWC, and *A*, but the inhibitory effect of Cd on stomatal conductance (g_s) and transpiration rate (*E*) was further promoted due to SA treatment. Cadmium-induced oxidative stress increased proline, lipid peroxidation and electrolyte leakage, but on exposure to SA, these parameters showed a marked decrease. Lower concentrations of Cd caused enhanced Cd transport into the plant. Cadmium suppressed the uptake of macro- and micro-nutrients, but exogenous application of SA restored the capability of plants to accumulate essential elements. SA mitigated the Cd-induced inhibition in the growth of mustard plants. Cadmium-induced increase in the activities of some key antioxidant enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) was reduced by the exogenous application of SA. This reflects that SA might have acted as one of the potential antioxidants in mustard plants under Cd stress.

Keywords: Antioxidants; Cadmium; Macronutrients; Mustard; Salicylic acid

1. Introduction

The distribution of cadmium (Cd) in the environment mainly takes place through human activities as well as mineralization processes of natural rocks enriched with metals including cadmium (Sanitadi et al., 1999). The addition of Cd to farming soils may occur mainly due to the use of phosphoric fertilizers (McLauglin et al., 2000). Cadmium is easily absorbed by roots and frequently transported to other plant parts. Like many other metals, it is highly toxic to living cells even at very low levels (Clemens et al., 1999). Since plants can easily absorb Cd, so it enters the food chain, thereby causing acute health disorders in humans. For instance, people consuming rice with relatively high levels of Cd may suffer from renal tubular disease (Watanabe et al., 1998). Cadmium affects the physio-biochemical activities of the living cells leading to phytotoxicity. Cadmium can suppress growth, induce leaf and root necroses and leaf chlorosis (Hernandez and Cooke, 1997), and perturbs chlorophyll (Chl) metabolism (Baryla et al., 2001), mineral nutrition (Moral et al., 1994; Ouzonidou et al., 1997), and water homeostasis (Poschenrieder et al., 1989). All these attributes can be effectively used as potential criteria of Cd toxicity in plants (Ernst et al., 2000). Cadmium toxicity can easily cause oxidative stress in plant cells, because Cd can effectively trigger the synthesis/accumulation of reactive oxygen species (ROS), which can cause cellular damage or lipid peroxidation (Shah et al., 2001) and inhibit or promote the activities of antioxidant enzymes involved in the oxidative defense system.

Salicylic acid (SA) acts as a signal involved in the expression of specific responses in plants to biotic and abiotic stresses. SA is involved in the protection of plants against multiple stresses like freezing (Janda et al., 1999; Tasgin et al., 2003), salinity

* Corresponding author. Tel.: +91 1933 219511, +91 9858376669 (Mobile); fax: +91 195 2234214.
E-mail address: pervaizbot@radiffmail.com (P. Ahmad).

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(Borsani et al., 2001), ozone and ultra-violet radiation (Sharma et al., 1996), water stress (Senaratna et al., 2000), and herbicides (Ananieva et al., 2004). There are many reports which show that SA can ameliorate the injurious effects of heavy metals on plants (Mishra and Choudhuri, 1999; Zhou et al., 2007, 2009). For example, exogenously applied SA ameliorated the adverse effects of Pb and Hg on cellular membranes in some rice cultivars (Mishra and Choudhuri, 1999). Popova et al. (2009) showed that exogenous application of salicylic acid can attenuate cadmium toxicity in pea seedlings. Such alleviating effect of SA was also examined in soybean seedlings grown under Cd stress (Drazic and Mihailovic, 2005). Protective effects of SA include up-regulation of anti-stress processes and recovery of growth processes after the stress is over (Sharikova et al., 2003). SA-induced protection of plants from oxidative injury caused by metals including Cd is mainly linked to enhanced accumulation of antioxidant enzymes (Wang et al., 2006). For example, Zhou et al. (2008) reported that Hg increased ROS levels and activated antioxidant systems in alfalfa plants.

Brassica juncea (Indian mustard) is one of the most important oil-seed crops grown extensively particularly in arid and semi-arid regions of the world (Ashraf and McNeilly, 2004; Singh et al., 2001). However, in most areas the growth and productivity of the crop are poor. This low economic yield can be attributed to the crop's susceptibility to a number of biotic and abiotic stresses including heavy metal stress. Metal stress adversely affects germination, growth, physiology and productivity of crops by causing ionic and osmotic stresses as well as oxidative damage (Larbi et al., 2002).

Owing to considerable evidence of the adverse effects of Cd on plant growth, it was hypothesized that SA can mitigate the injurious effects of cadmium on *B. juncea*. Thus, the primary objective of this work was to examine whether or not SA could mitigate the Cd-induced oxidative stress in mustard by regulating the antioxidant defense system and some key metabolites involved in stress tolerance.

2. Materials and methods

Seeds of *B. juncea* (L.) Czern. & Coss. cv. Varuna were surface sterilized in 5% solution of NaClO for 10 min. Ten sterilized seeds were sown into each plastic pot containing 5 kg well washed sand plus vermicompost (3:1) under glasshouse conditions. Mean day temperature was 28.6±5.1 °C, night temperature 16.3±7.6 °C, the photoperiod from 8 to 11 h, and average relative humidity (RH) 35.9±6.5 during the entire growth period. When the seedlings emerged from the sand, they were thinned to three per pot. Hoagland's nutrient solution (full strength) was supplied to all pots for 10 days and then different cadmium ($CdSO_4 \cdot 8H_2O$) treatments (0, 100 and 200 mg/l, i.e., 889.6 and 1779.2 μM, respectively) in Hoagland's nutrient solution were initiated. The Cd treatment solutions were applied every alternate day after leaching well the metal and nutrient solution already present in the sand. To maintain the moisture content of the sand, 200 ml of distilled water was applied to each pot every day. SA (1.0 mM) was sprayed in the evening with a manual sprayer (10 ml/plant) to plants mixed with tween-20 (a surfactant and spreading agent) every alternate day from the 1st day of treatment up to day 45. The experiment was laid out in a completely randomized design with five replicates. The plant leaves were collected for analysis after 45 days of application of SA.

2.1. Growth, Cd accumulation, gas exchange and RWC

Leaves and roots were separated, washed well with distilled water and fresh weights (FW) of the plant samples determined. For dry weight (DW) determination, the plant samples were dried at 70 °C for 48 h and then weighed. The Cd in the solution was determined using a Perkin-Elmer (Analyst Model 300) atomic absorption spectrophotometer. The heavy metal content was expressed as mg/g DW. Net CO_2 assimilation rate (A), stomatal conductance (g_s) and transpiration rate (E) of a fully expanded youngest leaf of each plant were measured using a portable infrared gas analyzer (LCA-4 model, Analytical Development Company, Hoddesdon, England). These measurements were made from 10:30 am to 12:00 noon. RWC of fully expanded third leaf from top per replicate was measured. Five leaf discs (each of 10 mm diameter) were excised from the interveinal areas of each leaf. For each replicate, 20 discs were pooled and their FW determined. The leaf discs were placed in distilled deionized water for 7 h under low irradiance and then the turgid weight (TW) recorded. Then the samples were dried at 80 °C for 24 h to determine DW. RWC was calculated using the following formula (Smart and Bingham, 1974):

$$RWC\,(\%) = (FW-DW \,/\, TW-DW) \times 100.$$

2.2. H_2O_2 content, lipid peroxidation (MDA) and electrolyte leakage assay

The hydrogen peroxide content was determined according to Velikova et al. (2000). Fresh fully expanded youngest leaf from top per replicate (500 mg) was homogenized with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 12,000 rpm for 15 min, and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI) solution. The absorbance of the supernatant was read at 390 nm. Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to Madhava Rao and Sresty (2000). Fresh fully expanded youngest leaf from top (500 mg) was homogenized with 2.5 ml of 0.1% trichloroacetic acid (TCA) solution. The extract was centrifuged at 10,000 rpm for 10 min. Four ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added to every 1 ml of the aliquot. After properly treating the mixture, it was centrifuged for 15 min at 10,000 rpm, and the absorbance of the supernatant was read at 532 nm. An extinction coefficient of 155/mM/cm was used for calculating MDA content. The total inorganic ions leaked out from the leaf tissues were determined following Dionisio-Sese and Tobita (1998). Twenty leaf discs were put in a boiling tube containing

10 ml of deionized water and electrical conductivity was measured (EC_0). The contents were heated at 50 and 60 °C for 25 min in a water bath and EC was measured (EC_1). Later, the contents were boiled at 100 °C for 10 min and the EC was again recorded (EC_2). The electrolyte leakage was calculated using the formula:

$$Electrolyte\,leakage\,(\%) = (EC_1 - EC_0) / (EC_2 - EC_0) \times 100.$$

2.3. Enzymatic antioxidants, SA and proline determination

SOD activity was appraised following Dhindsa and Matowe (1981). The assay solution consisting of 1.5 ml reaction buffer, 0.2 ml of methionine, 0.1 ml enzyme extract with equal amount of 1 M Na_2CO_3, 2.25 mM NBT solution, 3 mM EDTA, riboflavin and 1.0 ml of double distilled water, was placed under a 15 W florescent lamp for 10 min at 25/28 °C. Blank A containing the same reaction mixture, was placed in the dark. Blank B containing the same reaction mixture except for the enzyme extract was placed in light along with the sample. The reaction was terminated by turning off the light. A_{560} of each sample along with blank B was read against blank A and the difference in percent color reduction between blank B and the sample calculated. Fifty percent color reduction was considered as one unit of enzyme activity, and the activity was expressed as EU/mg protein. Catalase activity in the leaves was determined following Aebi (1984). Fresh biomass (500 mg) was homogenized in 5 ml of extraction mixture under cold conditions. The extract was centrifuged at 10,000 rpm for 20 min at 4 °C. Catalase activity was determined by examining the disappearance of H_2O_2 by measuring a dropping off in absorbance at 240 nm. The reaction was carried out in a final volume of 2 ml of reaction mixture containing reaction buffer with 0.1 ml of 3 mM EDTA, 0.1 ml of enzyme extract and 0.1 ml of 3 mM H_2O_2. The reaction was allowed to run for 10 min. Activity was calculated by using extinction coefficient (ε) 0.036/mM/cm and expressed in EU/mg protein. One unit of enzyme determined the amount necessary to decompose 1 μmol of H_2O_2 per min at 25 °C. Ascorbate peroxidase activity was appraised following Nakano and Asada (1981). Fresh fully expanded youngest leaf from top (1000 mg) of *B. juncea* was ground in 4 ml extraction buffer and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for the assay immediately. The assay was performed in 1.0 ml reaction buffer in the presence of 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and enzyme extract. APX activity was calculated as the decrease in A_{290} nm of ascorbate using an extinction coefficient of 2.8/mM/cm, and expressed in EU/mg protein. One unit enzyme was considered as the amount necessary to decompose 1 μmol of substrate per min at 25 °C. Glutathione reductase activity was determined following Foster and Hess (1980). The reaction mixture (1 ml) consisted of enzyme extract (0.1 ml), 100 mM potassium phosphate buffer (0.75 μl) (pH 7.0) containing 1.0 mM EDTA, 150 μM NADPH and 500 μM oxidized glutathione. The enzyme activity was determined at 340 nm. An extinction coefficient for NADPH of 6.22/mM/cm was used to determine GR activity and expressed as μmol NADPH oxidized/mg protein/min. Fresh leaves (1.0 g) were ground well in 4 ml of methanol. The homogenate was centrifuged at 20,000 rpm and 4 °C for10 min. The extracts were vacuum-dried at room temperature and then dissolved in 300 ml of 20 mM potassium phosphate buffer (pH 7.6). SA concentrations were determined following Siegrist et al. (2000) and Metwally et al. (2003) with an HPLC system equipped with fluorescence detector (LC-2010 AHT, SHIMADZU, Japan). Proline concentration in the leaves was determined spectrophotometerically following Bates et al. (1973). Fresh leaf material (300 mg each sample) was ground in 10 ml of 3% aqueous sulfosalicylic acid. The supernatant obtained after centrifugation at 12,000 rpm for 15 min was mixed with an equal volume of acetic acid and acid ninhydrin and incubated for 1 h at 100 °C. The chromatophore containing toluene was then aspirated from the aqueous phase, and its absorbance read spectrophotometrically at 520 nm (Beckman 640 D, USA) using toluene as a blank.

2.4. Determination of inorganic nutrients

Dried shoot and root material (100 mg) was powdered and digested in H_2SO_4/HNO_3 mixture (1/5, v/v) for 24 h, and then it was treated with $HNO_3/HClO_4$ mixture (5/1, v/v). The element concentration was measured by atomic absorption spectrophotometer (Analyst 300, Perkin-Elmer, Germany).

2.5. Statistical analysis

Data for each variable were subjected to one way analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) at 5% probability was employed for assessing the significant differences among the mean values of different attributes. The values are means of five replications.

3. Results

The results related to the influence of Cd and SA on biomass production are presented in Fig. 1. Exposure of mustard plants to high Cd levels resulted in a marked reduction in fresh weights of both shoot and roots (15.8% and 33.7% reduction in shoot biomass at 100 and 200 mg/l Cd, respectively). The root fresh weight was also decreased to 27.1% at 100 mg/l Cd and 46.6% at 200 mg/l Cd. Dry weight of shoot and root also showed a decline under Cd stress. The decrease was observed to be 30.8% in shoots and 33.9% in roots at 200 mg/l Cd. However, a substantial improvement in shoot fresh and dry weights was noticed due to exogenously applied salicylic acid under both Cd regimes. Increase in shoot fresh weight (7% relative to that in Cd treatment) and root fresh weight (5%) was observed on application of 1.0 mM SA under 200 mg/l Cd. Shoot and root dry weight also showed a 5% increase at the same concentration of Cd and SA.

Mustard seedlings accumulated relatively higher amount of Cd in the roots than that in the shoots. Cd contents of root and shoot tissues were very low when the growth medium lacked Cd, but in contrast, a substantial increase in Cd accumulation

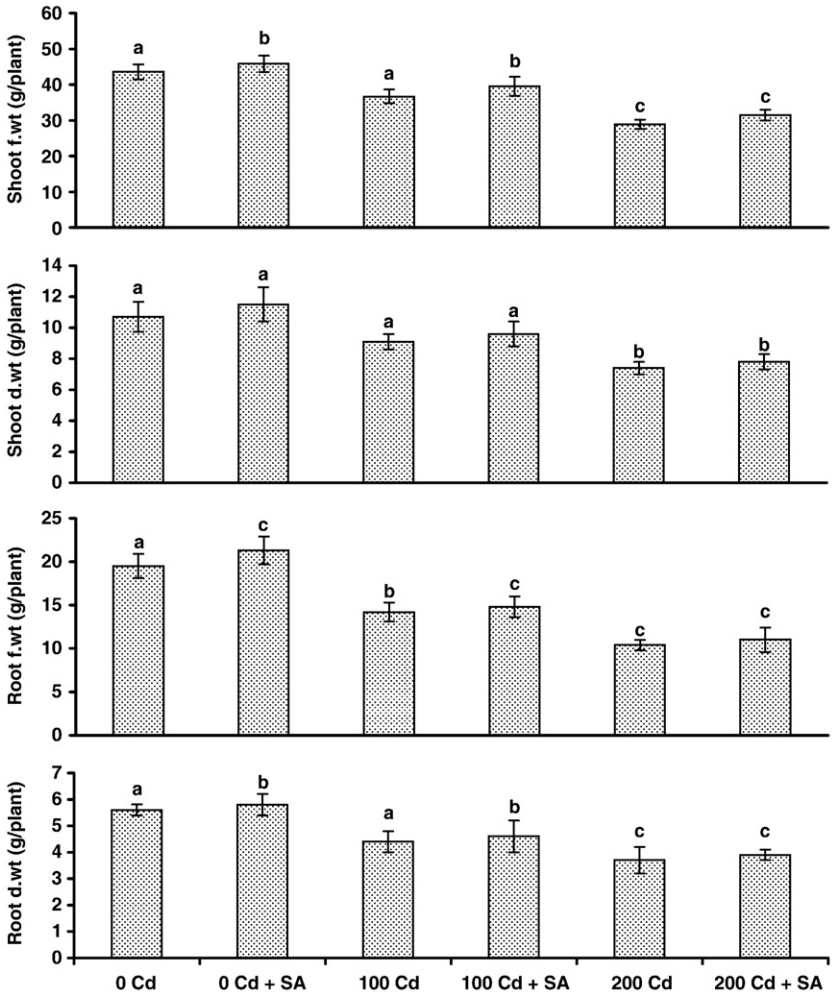

Fig. 1. Effect of Cd and SA on shoot and root fresh and dry weights of mustard plants. Means with different letters (a–c) within each attribute are statistically different at 5% level (mean±S.E.; $n=5$).

was noticed in plants fed with 100 or 200 mg/l Cd. However, exogenous application of SA showed inconsistent effect with respect to Cd content in shoot and root (Table 1). The growth retardation of mustard plants by Cd treatment was found to be associated with a decrease in photosynthetic rate (A). A substantial decline in A was observed at 200 mg/l Cd. Exogenous application of SA mitigated the injurious effects of Cd on A (Table 1). Stomatal conductance (g_s) was also altered by Cd treatment. Application of SA allayed the negative effect of Cd on g_s. The influence of Cd and SA on transpiration rate (E) was the same as observed for g_s (Table 1).

RWC was decreased in mustard seedlings on exposure to Cd treatment. However, application of SA assuaged the adverse effects of Cd on this attribute (Table 1).

Table 1
Effect of Cd (100 and 200 mg/l) and SA (1.0 mM) on accumulation of salicylic acid, shoot and root Cd, CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E), proline, RWC, and electrolyte leakage in mustard seedlings (means±S.E; $n=5$).

	0 Cd	0 Cd+SA	100 Cd	100 Cd+SA	200 Cd	200 Cd+SA
Leaf SA (μg/g FW)	30.9±2.6a	32.4±2.9a	413±3.2b	316±3.3c	531±3.5d	478±3.6d
Shoot Cd (μmol/g DW)	nd	nd	8.05±0.66a	8.45±0.69a	13.11±0.11b	12.02±0.15b
Root Cd (μmol/g DW)	nd	nd	16.23±1.5a	16.95±1.7a	23.21±1.9b	22.27±1.8b
A (μmol CO_2 m^{-2} s^{-1})	12.4±0.53a	8.6±0.17b	11.9±0.19a	9.1±0.23c	7.8±0.21d	8.8±0.15d
g_s (mmol m^{-2} s^{-1})	0.35±0.06a	0.45±0.08a	0.32±0.08a	0.35±0.04a	0.54±0.05b	0.25±0.02a
E (mmol H_2O m^{-2} s^{-1})	5.6±0.05a	5.0±0.04a	5.4±0.04a	4.5±0.03b	3.9±0.03c	3.0±0.02d
Proline (μg g^{-1} FW)	56.01±2.2a	95.73±5.7b	58.04±4.4a	104.8±8.1c	112.5±8.5d	133.7±9.8e
RWC	90.5±5.2a	60.7±5.4b	92.1±5.2a	69.6±5.9c	40.4±4.1d	45.6±3.8d
Electrolyte leakage (%)	8.3±0.67a	19.5±0.19c	7.9±0.32b	14.7±0.11d	28.3±0.15e	21.5±0.17e

nd=not detectable.
Means with different letters (a–e) within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level.

Hydrogen peroxide (H_2O_2) content showed approximately 48.7% and 61.1% rise at 100 and 200 mg/l Cd, respectively. However, exogenously applied SA caused a substantial decrease of 42.8% and 53.4% at the same concentrations of Cd (Fig. 2). Lipid peroxidation was appraised in terms of MDA content. The MDA content was observed to be 19.5% at 100 mg/l Cd and 42.8% at 200 mg/l Cd. Lipid peroxidation reduced by 14.8% and 15.1% on foliar application of SA to 100 and 200 mg/l Cd-treated plants, respectively (Fig. 2). Electrolyte leakage increased significantly under both Cd treatments. SA application prevented to some extent Cd-induced electrolyte leakage (Table 1).

The influence of Cd and SA on antioxidants is shown in Fig. 3. The SOD content increased with the increase in exogenous Cd level, and the increase was observed to be 15.4% and 26.5% at 100 and 200 mg/l Cd, respectively. However, exogenous application of SA caused a significant decline in SOD activity in Cd-treated plants. CAT activity decreased under Cd stress (Fig. 3), being more marked at 200 mg/l Cd. However, exogenous application of SA further decreased CAT activity in Cd-treated plants. Ascorbate peroxidase (APX) activity was stimulated by Cd toxicity as compared to that in the control (Fig. 3). Maximum APX activity (12.8%) was observed at 200 mg/l Cd. SA application reduced the activity of APX in Cd-treated plants, being more marked at 100 mg/l Cd. Glutathione reductase (GR) also increased by Cd stress. The magnitude of increase in GR activity was 17.3% at 200 mg/l Cd. Exogenously applied SA reduced the levels of GR in all Cd-treated plants.

SA was found in small quantity in the leaves of control plants, but on exposure to Cd (100 and 200 mg/l) the level of SA increased significantly, being more prominent in 200 mg/l Cd-treated plants. However, exogenously applied SA significantly reduced intrinsic SA levels in Cd-treated plants (Table 1). Exposure of mustard seedlings to Cd increased proline content. The maximum increase in proline (50.2%) was at 200 mg/l Cd. However, exogenous application of SA further enhanced the amount of proline in mustard plants exposed to Cd stress (Table 1).

Cadmium caused a marked reduction in shoot and root contents of S, Mn, Mg and K in the present study. For example, S content decreased with the increase in the concentration of Cd, but foliar applied SA mitigated the negative effects of Cd by restoring the S content in both shoots and roots. The contents of Mn and Mg decreased to 50.8% and 30.7% in roots and 39.7% and 44.7% in shoots, respectively at 200 mg/l Cd (Table 2). Exogenous application of SA helped the plants to restore these elements. Cadmium did not induce significant changes in Ca concentrations in the roots, but in the shoot tissue a 30.1% decrease in Ca was observed at 200 mg/l Cd. SA application increased the Ca content in both shoots and roots. Cadmium caused 10% and 31.8% decrease in K content in the roots and

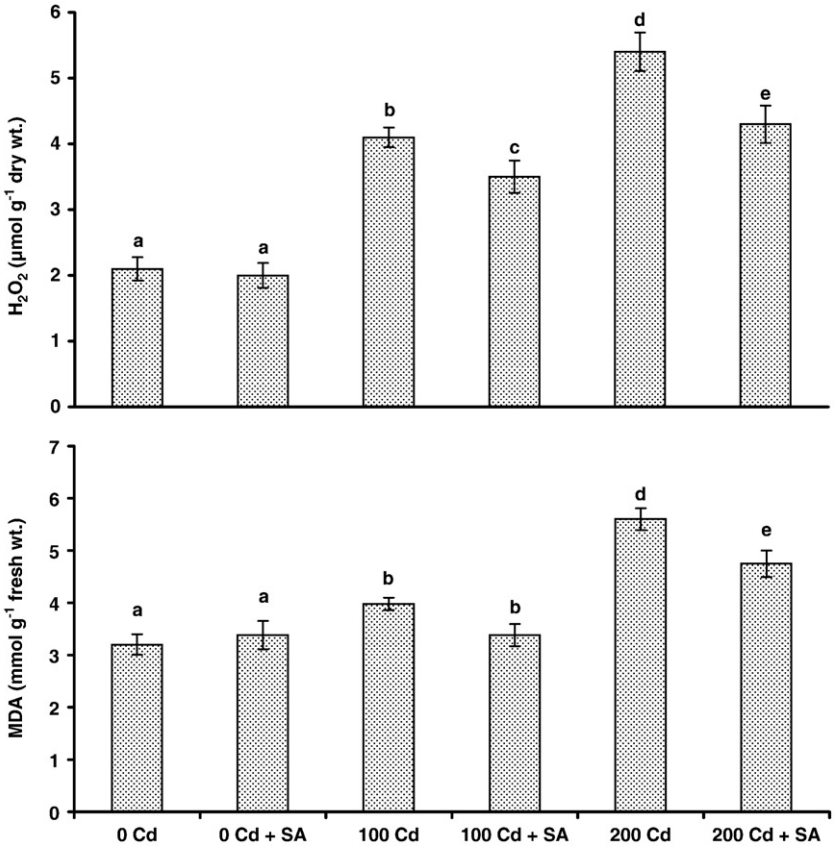

Fig. 2. Effect of Cd and SA on hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) of mustard plants. Means with different letters (a–e) within each attribute are statistically different at 5% level (mean±S.E.; $n=5$).

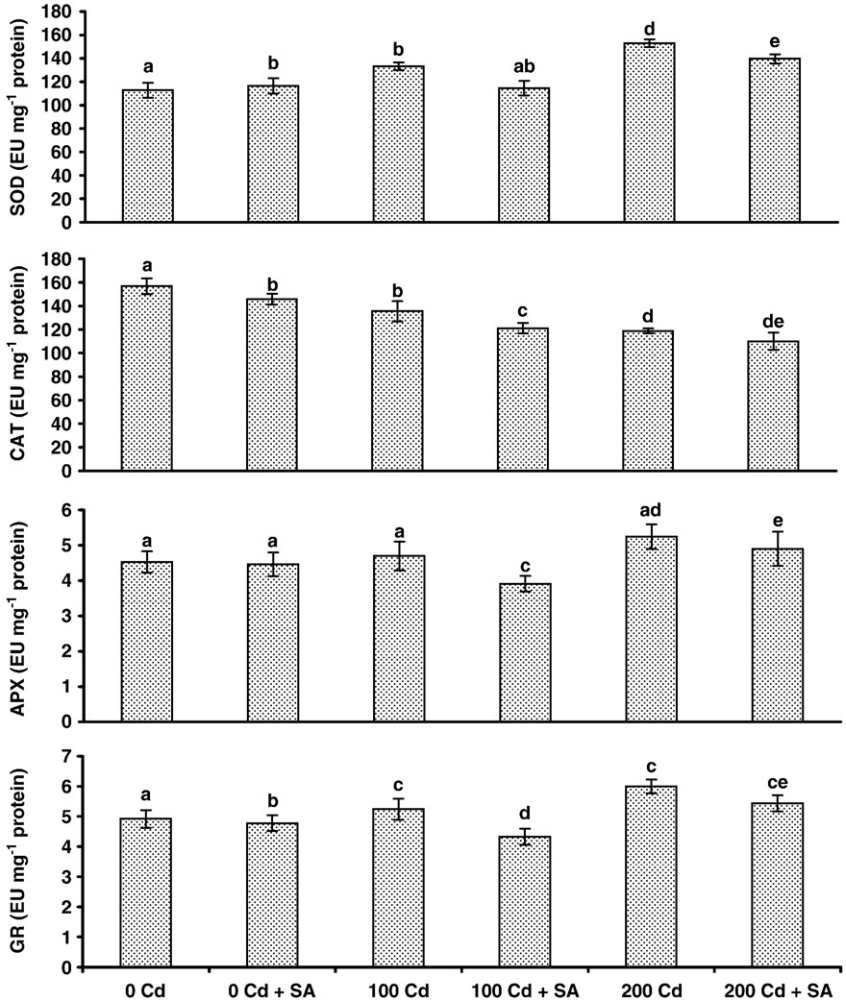

Fig. 3. Effect of Cd and SA on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) of mustard plants. Means with different letters (a–e) within each attribute are statistically different at 5% level (mean±S.E.; $n=5$).

shoots, at 200 mg/l, respectively, however, a significant restoration in K content was observed by foliar application of SA.

4. Discussion

In the present study, exposure to high Cd level in the growth medium resulted in decreased shoot and root fresh mass of mustard plants. The growth inhibition was found to be associated with Cd-induced decrease in net photosynthesis and water-use efficiency. Growth inhibition by Cd could have been due to the inhibition in cell division and elongation rate of cells that mainly occur by an irreversible inhibition of proton pump responsible for the process (Liu et al., 2004). Similar to our study, Cd application reduced fresh weights of shoots and roots close to 50%, and dry weights of shoots and roots by about

Table 2
Effect of Cd (100 and 200 mg/l) and SA (1.0 mM) on mineral nutrients of shoots and roots of mustard seedlings (mean±S.E; $n=5$).

Mineral nutrients (μmol g^{-1} d. wt.)	0 Cd	0 Cd+SA	100 Cd	100 Cd+SA	200 Cd	200 Cd+SA
Shoot S	108±3.5a	99.34±2.9c	116±3.1b	104±2.1ab	88.2±1.9c	100.3±1.7d
Shoot Mn	17.5±1.3a	15.43±1.3c	23.5±1.6b	12.64±1.7c	10.55±1.2d	7.97±1.1e
Shoot Mg	205±4.2a	298±4.3b	275±4.5b	215±3.8ab	371±4.7c	286±3.9bd
Shoot Ca	93±2.5a	75±3.7b	97±3.3a	78±4.1b	65±4.9c	69±5.1c
Shoot K	415±6.2a	307±6.7c	495±5.3b	385±3.4d	283±2.7e	315±2.9 cd
Root S	317±5.1a	295±4.5b	318±5.2a	302±2.5b	254±2.7c	294±2.9d
Root Mn	59±2.1a	42±1.9c	63±2.5b	31±1.5c	29±1.3 cd	15±1.1d
Root Mg	663±9.7a	863±8.3c	770±8.5b	735±6.1b	958±8.4d	845±7.7c
Root Ca	159±2.2a	168±2.5a	162±2.1a	170±2.7b	173±3.5b	178±3.3c
Root K	1250±20.4a	1012±18.4c	1396±24.1b	1152±19.5c	925±15.2d	1117±18.8e

Means with different letters (a–e) within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level.

35% in barley (Metwally et al., 2003). Ameliorative impact of SA on the growth of mustard plants under Cd stress as observed in the present study has already been reported in different crop plants under abiotic stress conditions and this was ascribed to the role of SA in nutrient uptake (Glass, 1974), water relations (Barkosky and Einhellig, 1993), stomatal regulation (Arfan et al., 2007), and photosynthetic capacity and growth (Arfan et al., 2007; Khan et al., 2003; Popova et al., 2009). However, in the present investigation, exogenous application of salicylic acid improved shoot fresh weight of mustard plants exposed to Cd stress. This could have been due to the reason that Cd is retained in roots and a very small amount of it is transported to shoots (Caltado et al., 1983). Choudhury and Panda (2004) reported that Cd content in SA non-primed rice roots was higher as compared to that in SA primed roots. The SA-induced differential accumulation of Cd was considered as one of the potential physiological effects of SA on plants (Choudhury and Panda, 2004). SA is also known to reduce the accumulation of heavy metals other than Cd (Yang et al., 2003). SA caused reduction in Cd uptake in hemp (*Cannabis sativa*) and hence alleviated Cd toxicity (Shi et al., 2009). It has been observed that alteration in photosynthesis with exogenous SA under environmental stresses could be due to either non-stomatal or stomatal factors (Athar and Ashraf, 2005; Dubey, 2005). However, SA application was found to be effective in improving the rate of photosynthesis in Cd-treated mustard plants. Application of SA can recede ABA-induced stomatal closure (Rai et al., 1986). While, the SA-induced reduction in g_s and E in mustard plants are parallel to those of Larque-Saavedra (1979) who showed that exogenously applied SA had an anti-transpiration effect on the leaves of *Phaseolus vulgaris* which may be due to the reduction in stomatal conductance of epidermal strips e.g., *Commelina communi* (Larque-Saavedra, 1979).

Hydrogen peroxide (H_2O_2) production increased markedly in mustard plants upon Cd treatment. Increase in H_2O_2 content is also reported in other plants upon Cd treatment (Kuo and Kao, 2004; Olmos et al., 2003). SA treatment decreased the level of H_2O_2 and led to reduce Cd-induced oxidative injuries in mustard plants in the present study. Similar results have also been recently reported by Popova et al. (2009) that a small decrease in H_2O_2 took place upon SA treatment in pea seedlings exposed to Cd stress. The SA-induced reduction in H_2O_2 content in Cd-treated plants may have been due to its effect as an antioxidant in counteracting to some extent the generation of H_2O_2 under Cd stress. Accumulation of MDA, a product of lipid peroxidation, is commonly used as one of the potential indicators of oxidative stress (Taulavuori et al., 2001; Skórzyńska-Polit and Krupa, 2006). Monteiro et al. (2009) observed increased MDA content due to Cd stress in lettuce. Furthermore, Zhang et al. (2007) also demonstrated increased MDA content in the leaves of *Bruguiera gymnorrhiza* exposed to a variety of metals, and suggested lipid peroxidation as a prospective biomarker of metal stress. The Cd-induced enhancement in MDA content in the mustard plants decreased due to SA application. These findings are parallel to what Metwally et al. (2003) observed that pretreatment of barley seeds with SA decreased the MDA content.

Cadmium-induced oxidative stress leads to increased activities of antioxidative enzymes in plants (Chaoui et al., 1997; Metwally et al., 2003). For example, an increase in total SOD, APX and GR activity was found after the application of Cd in *Bacopa monnieri* (Mishra et al., 2006), *Triticum aestivum* (Khan et al., 2007), and *Brassica juncea* (Mobin and Khan, 2007), whereas CAT activity was declined in *Bacopa monnieri* (Mishra et al., 2006), and *Phragmites australis* (Iannelli et al., 2002). In the present study, Cd toxicity promoted the activity of antioxidant enzymes except that of CAT and exogenous application of SA resulted in reducing the activities of all four antioxidant enzymes in the Cd-treated plants. SA is an iron chelating molecule which can directly scavenge hydroxyl radicals (Dinis et al., 1994). The Cd-induced high levels of SA in the mustard plants may have functioned directly as a prospective antioxidant to counteract the ROS. In another study, Guo et al. (2007) reported that exogenously applied SA improved rice Cd tolerance by accelerating the activities of enzymes involved in the antioxidant defense system. Several reports show that SA can induce antioxidant activity under multiple stresses such as UV, heat, and salt (Janda et al., 1999; Mishra and Choudhuri, 1999; Tissa et al., 2000). Mba et al. (2007) also observed that the activity of SOD in cabbage increases with the increase in external concentration of Cd, but the SA treatment in the culture reduced the activity of SOD. Zawoznik et al. (2007) showed that in SA-deficient *Arabidopsis* plants, catalase activities significantly increased. These results are in agreement with those of Ding et al. (2002) who showed that CAT activity was suppressed in the fruits of tomato by SA treatment.

Glutathione reductase (GR) is known to catalyze some vital steps of the ascorbate–glutathione cycle (Noctor and Foyer, 1998). The enzyme maintains high ratio of GSH/GSSG, which is essentially required for the regeneration of ascorbate and for the activation of a number of enzymes involved in CO_2-fixation (Noctor and Foyer, 1998). GR activity increased upon Cd treatment in the present study, but it was suppressed by SA treatment. Similar results were observed by Metwally et al. (2003) in barley seedlings. In the present study, Cd application caused a marked increase in intrinsic SA content in the leaves of mustard plants. Similar Cd-induced increase in SA has been found in maize (Krantev et al., 2009; Pal et al., 2005), and pea (Popova et al., 2009). However, exogenous application of SA to Cd-treated plants resulted in decreased accumulation of intrinsic SA in Cd-treated mustard plants. The beneficial effect of exogenously applied SA can be observed in consequent changes in a number of biochemical attributes. For example, proline increased markedly in the Cd-treated plants and SA application further enhanced the proline levels in these plants. Enhanced proline accumulation in response to Cd toxicity has been earlier demonstrated (Dhir et al., 2004) in *Triticum aestivum*, *Vigna radiata* and sunflower (Zengin and Munzuroglu, 2006). Thus, proline accumulation is a potential indicator of stress tolerance (Ashraf and Foolad, 2007).

Cadmium application can lead to a deficiency of macro- and micro-nutrients in plants (Hernandez and Cooke, 1997; Larbi et al., 2002; Ramos et al., 2002), which may cause other

changes in plant metabolism. Cadmium significantly disturbs ionic homeostasis, but SA can overcome this perturbance. For example, Cd-induced decrease in K content may have been due to decreased K uptake caused by the antagonistic effect of Cd (Hernandez and Cooke, 1997; Murphy et al., 1999). Magnesium, sulphur and manganese concentrations in the leaves of mustard plants decreased, but the exogenous application of SA attenuated the negative effect of Cd on these nutrients.

5. Conclusion

SA-induced alleviation of the negative effects of Cd toxicity may have been due to the following reasons. SA-treated mustard plants accumulated considerably higher levels of Cd in the roots as compared to those in the shoot which might have one of the effective strategies of these plants to check the uptake of Cd to upper plant parts i.e. shoot and hence promote shoot growth. SA also allayed the Cd-induced oxidative damages. The values of MDA, H_2O_2 and relative water content of SA-treated plants were considerably lower than those in the Cd-treated plants. Thus, the adverse effects of Cd toxicity on growth, enzymes involved in the oxidative defense mechanism and nutrient uptake in mustard plants can be alleviated by foliar spray of SA.

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