Salicylic Acid Mitigated Cadmium Toxicity by Attenuating the Oxidative Stress in Pea (*Pisum sativum* L.) Plants

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Abstract— The plants of pea were grown in the presence of $CdCl_2$ (0.75 and 1.50 mM Cd^{2+}), were sprayed with 2.0 mM of salicylic acid (SA) at 10 days after sowing (DAS) and were sampled at 49 DAS. The plants exposed to Cd^{2+} exhibited a significant decline in growth, pigment content, relative water content (RWC) and inorganic nutrients content. However, the follow up treatment with SA mitigated the stress generated by Cd^{2+} and significantly improved the aforesaid parameters. The Cd^{2+} increased proline content, electrolyte leakage, hydrogen peroxide, lipid peroxidation and plant Cd^{2+} content. However, the SA treatment attenuated the adverse effects of Cd^{2+} on these attributes. Cd^{2+}-induced increase in the activities of some key antioxidant enzymes, superoxide dismutase, ascorbate peroxidase, catalase, and glutathione reductase was reduced by the exogenous application of SA. This reflects that SA might have acted as one of the potential antioxidants in pea plants under Cd^{2+} stress.

Keywords: Antioxidants; Cadmium; Growth; Nutrients; Peas; Salicylic acid

I. Introduction

PEA (*Pisum sativum* L.) is one of the popular vegetable crops in Egypt. It acts as a rich source in protein, carbohydrates and nutrients for human diet. Pea is widely cultivated on newly-reclaimed soils in Egypt. However, the newly-reclaimed soils need extensive use of fertilizers including phosphoric fertilizers. The addition of cadmium (Cd^{2+}) to farming soils may occur mainly due to the use of phosphoric fertilizers McLauglin et al.,[1], and through human activities as well as mineralization processes of natural rocks enriched with metals including cadmium Sanitadi et al.,[2]. Cd^{2+} 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., [3]Since plants can easily absorb Cd^{2+}, so it enters the food chain, thereby causing acute health disorders in humans. Cd^{2+} affects the physiobiochemical activities of the living cells leading to phytotoxicity. Cd^{2+} can suppress growth, induce leaf and root necroses and leaf chlorosis Hernandez and Cooke,[4], and perturbs chlorophyll metabolism(Baryla et al.,[5] , mineral nutrition(Ouzonidou et al., [6], and water homeostasis Poschenrieder et al.,[7]. All these attributes can be effectively used as potential criteria of Cd^{2+} toxicity in plants Ernst et al.,[8].

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Cd^{2+} toxicity can easily cause oxidative stress in plant cells, because Cd^{2+} can effectively trigger the synthesis/accumulation of reactive oxygen species (ROS), which can cause cellular damage or lipid peroxidation Shah et al.,[9] 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 Tasgin et al.,[10], salinity Borsani et al.,[11] , ozone and ultra-violet radiation Sharma et al.,[12]), water stress Senaratna et al.,[13]), and herbicides Ananieva et al.,[14]. There are many reports which show that SA can assuage the injurious effects of heavy metals on plants Mishra and Choudhuri,[15]; Zhou et al.,[16]&[17]. For example, exogenously applied SA attenuated the adverse effects of Pb and Hg on cellular membranes in some rice cultivars[15]. Popova et al. [18]showed that exogenous application of SA can mitigate Cd^{2+} toxicity in pea seedlings. Such alleviating effect of SA was also examined in soybean seedlings grown under Cd^{2+} stress Drazic and Mihailovic,[19]. Protective effects of SA include up-regulation of anti-stress processes and recovery of growth processes after the stress is over Sharikova et al.,[20]. SA-induced protection of plants from oxidative injury caused by metals including Cd^{2+} is mainly linked to enhanced accumulation of antioxidant enzymes Wang et al.,[21]. For example, Zhou et al.[16] reported that Hg increased ROS levels and activated antioxidant systems in alfalfa plants.

Owing to considerable evidence of the adverse effects of Cd^{2+} on plant growth, it was hypothesized that SA can assuage the injurious effects of Cd^{2+} on *Pisum sativum*. Thus, the primary objective of this work was to examine whether or not SA could mitigate the Cd^{2+}-induced oxidative stress in pea by regulating the antioxidant defense system and some key metabolites involved in stress tolerance.

II. Materials And Methods

Plant material and growth conditions

The seeds of pea (*Pisum sativum* L., cv. Master-B) were obtained from the Agricultural Research Center (Department of Vegetable Crops, Giza, Egypt). The health seeds were surface sterilized in 5% solution of NaClO for 5-10 min. Three sterilized seeds were sown, on 5 November 2009 and 2010, in each plastic pot (40 cm in diameter, 50 cm in deep) filled with acid then deionized water washed sand. Pots were arranged for growing plants in an open greenhouse. The average day and night temperatures were 18 ± 3 ^{o}C and 10 ± 2

^{o}C, respectively. The relative humidity ranged from 60.4 to 65.2%, and day-length from 11 to 12 h. Hoagland's nutrient solution (full strength) was supplied to all pots for 10 days and then different cadmium (Cd^{2+}) treatments (0, 0.75 and 1.50 mM) using cadmium chloride in Hoagland's nutrient solution were initiated. Salicylic acid (SA, 2.0 mM) solution was sprayed in the evening with a manual sprayer to plants (to run off) mixed with tween-20 (a surfactant and spreading agent) every 3 days directly after the irrigation up to week 7. The selection of the concentration was based on preliminary studies (data not shown). The concentration of SA (2.0 mM) used generated the best response. Therefore, it was selected for the experiments. With regard to the concentrations of Cd^{2+}, the concentrations above 1.50 mM proved lethal (at the end of the preliminary experiment; day 49). Therefore, the concentrations below the lethal concentration (i.e. 0.75 and 150 mM) were used in these experiments. The experiments were laid out in a completely randomized design with six replicates. The plants were collected for assess all attributes after 39 days of application of SA.

Growth, SA, Cd and photosynthetic pigments determinations

Shoots 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.

SA content (mg kg^{-1} FW) of fully expanded third leaf from top per replicate was determined following Siegrist et al. [22]and Metwally et al.[23] with an HPLC system equipped with fluorescence detector (LC-2010 AHT, SHIMADZU, Japan).

The powdery dried plant parts (i.e. shoots and roots) were used to determine their content of cadmium by using a Perkin-Elmer, Model 3300, Atomic Absorption Spectrophotometer (Chapman and Pratt,[24]. Total chlorophyll and carotenoids contents (mg g^{-1} FW) were estimated adopting the procedure given by Arnon[25]. Leaf discs were homogenized with 80% acetone and centrifuged; the optical density of the acetone extract was measured at 663, 645 and 470 nm using a UV-160A UV Visible Recording Spectrometer, Shimadzu, Japan.

Proline and relative water contents, and electrolyte leakage determinations

Proline content in fully expanded third leaf from top per replicate was measured by rapid colorimetric method as suggested by Bates et al.[26]. Proline was extracted from 0.5 g of dry leaf samples by grinding in 10 ml of 3% sulphosalicylic acid and the mixture was then centrifuged at 10,000 ×g for 10 min. Two ml of the supernatant was added into test tubes to which 2 ml of freshly prepared acid-ninhydrin solution was added. Tubes were incubated in a water bath at 90 ºC for 30 min. The reaction was terminated in ice-bath. The reaction mixture was extracted with 5 ml of toluene and vortexed for 15 s. The tubes were allowed to stand at least for 20 min in darkness at room temperature to allow the separation of toluene and aqueous phase. The toluene phase was then carefully collected into test tubes and toluene fraction was read at 520 nm. The proline concentration in the sample was determined from a standard curve using analytical grade proline and calculated on dry weight basis (as mg kg^{-1} DW).

The relative water content (RWC) of fully expanded third leaf from top per replicate was determined in fresh leaf discs of 2 cm^2 diameter, excluding midrib. Discs were weighed quickly and immediately floated on DDW in Petri dishes to saturate them with water for the next 24 h, in dark. The adhering water of the discs was blotted and turgor mass was noted. Dry mass of the discs was recorded after dehydrating them at 70°C for 48 h. RWC was calculated by placing the values in the following formula:

$$RWC = \frac{\text{Fresh mass - dry mass}}{\text{Turgor mass - dry mass}} \times 100$$ (Hayat et al., [27]

The total inorganic ions leaked out from the leaf tissues were determined following Dionisio-Sese and Tobita[28]. 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 (%) = $(EC_1 - EC_0) / (EC_2 - EC_0) \times 100$

Hydrogen peroxide, lipid peroxidation (MDA) and antioxidant enzymes assays

The hydrogen peroxide (H_2O_2) content was determined according to Velikova et al.[29]. Fresh fully expanded third 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[30]. Fresh fully expanded third leaf from top (0.5 g) 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^{-1} cm^{-1} was used for calculating MDA content.

Superoxide dismutase (SOD) activity in the leaves was appraised following Dhindsa and Matowe[31]. 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. A560 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^{-1} protein.

Catalase (CAT) activity in the leaves was determined following Aebi[32]. 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. CAT 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^{-1} cm^{-1}, and expressed in EU mg^{-1} protein. One unit of enzyme determined the amount necessary to decompose 1 µmol of H_2O_2 per min at 25 °C.

Ascorbate peroxidase (APX) activity in leaves was appraised following Nakano and Asada[33]. Fresh fully expanded youngest leaf from top (1000 mg) of *Pisum sativum* 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 A290 nm of ascorbate using an extinction coefficient of 2.8 mM^{-1} cm^{-1}, and expressed in EU mg^{-1} protein. One unit enzyme was considered as the amount necessary to decompose 1 µmol of substrate per min at 25 °C.

Glutathione reductase (GR) activity was determined following Foster and Hess[34]. 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^{-1} cm^{-1} was used to determine GR activity and expressed as µmol NADPH oxidized mg^{-1} protein min^{-1}. 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).

Determination of macro- and micronutrients

Shoot and root N contents (mg g^{-1} DW) were determined according to Hafez and Mikkelsen[35]. An Orange-G dye solution was prepared by dissolving 1.0 g of 96% (w/w) assay-dye in 1.0 l of distilled water with 21.0 g citric acid, which acted as a buffer to maintain the correct pH, and 2.5 ml 10% (v/v) thymol in alcohol as an inhibitor of microbial growth. Ground plant sample material (0.2 g) was placed in a centrifuge tube and 20 ml of the dye reagent solution was added. The contents of the tube were shaken for 15 min. After filtration, the solution was diluted 100-times with distilled water and its absorbance was measured at 482 nm. N contents were calculated using the formulae:

N (%) = 0.39 + 0. 954 × Dye absorbed (g /100 g), and

Dye absorbed (g /100 g) = $(a - b / a)$ $(cfv / w) \times 100$

where, *a* was the absorbance of the dye reagent solution at 482 nm without any plant material (blank), *b* was the absorbance of the dye reagent solution at 482 nm with plant material, *c* was the concentration of the dye reagent (1.0 g l^{-1} distilled water), *f* was the purity factor of the dye reagent (96%), *v* was the volume of the dye reagent solution used per sample (20 ml), and *w* was the weight of ground dry material in g (0.2).

The powdery dried plant parts (i.e. shoots and roots) were also used to determine their contents of other studied nutrients by using a Perkin-Elmer, Model 3300, Atomic Absorption Spectrophotometer Chapman and Pratt, [24]

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 six replications.

III. Results

Effect of Cadmium (Cd^{2+}) and Salicylic acid (SA) on biomass production

The results related to the impact of Cd^{2+} and SA on fresh and dry weights of pea shoots and roots are presented in Table 1. Exposure of plants to both Cd^{2+} levels resulted in a marked reduction in fresh weights of both shoots and roots. The shoot fresh weight was decreased to 15.1% at 0.75 mM Cd and 35.8% at 1.50 mM Cd^{2+}. The root fresh weight was reduced to 26.8% and 46.5% at 0.75 and 1.50 mM Cd^{2+}, respectively. Dry weight of shoot and root also showed a significant decline under Cd^{2+} stress. The decrease was observed to be 32.1% in shoots and 38.5% in roots at 1.50 mM Cd. However, a substantial improvement in shoot fresh and dry weights was noticed due to exogenously applied SA under both Cd^{2+} regimes. Increase in shoot fresh weight (12.7% relative to that in Cd^{2+} treatment) and root fresh weight (15.8%) was observed on application of 2.0 mM SA under 1.50 mM Cd. Shoot and root dry weights also showed 7.3% and 16.7% increases, respectively at the same concentrations of Cd^{2+} and SA.

Effect of Cd^{2+} and SA on endogenous SA, Cd^{2+}, photosynthetic pigments, proline, relative water content (RWC) and electrolyte leakage (EL)

SA was found in small quantity in the leaves of control plants, but on exposure to Cd^{2+} (0.75 and 1.50 mM) the level of SA significantly increased, being more prominent in 1.50 mM Cd^{2+}-treated plants. However, exogenously applied SA significantly reduced endogenous SA levels in Cd^{2+}-treated plants (Table 2). Pea plants accumulated relatively higher amount of Cd^{2+} in the roots than that in the shoots. Cd^{2+} contents of root and shoot tissues were not detected when the growth medium lacked Cd^{2+}. In contrast, a substantial increase in Cd^{2+} accumulation was noticed in plants treated with 0.75 or 1.50 mM Cd^{2+}. Cd^{2+} treated-plants exogenously applied with SA showed a significant decrease in the Cd^{2+} content in both shoots and roots (Table 2). The growth retardation of pea plants by Cd^{2+} application was found to be associated with a significant decrease in total chlorophyll content. A substantial decline in total chlorophyll was observed at 1.50 mM Cd^{2+}.

Exogenous application of SA mitigated the injurious effects of Cd^{2+} on chlorophyll (Table 2). Carotenoids content was also altered by Cd^{2+} treatment. Application of SA allayed the negative effect of Cd^{2+} on carotenoids (Table 2). Exposure of pea plants to Cd^{2+} increased proline content. The maximum increase in proline (97.2%) was at 1.50 mM Cd^{2+}. However, exogenous application of SA further increased the amount of proline (138.8%) in pea plants exposed to the same concentration of Cd^{2+} (Table 2). RWC was decreased in pea plants on exposure to Cd^{2+} treatment, particularly in the plants treated with 1.50 mM Cd^{2+}. However, the application of SA attenuated the adverse effects of Cd^{2+} on this attribute (Table 2). Electrolyte leakage increased significantly under both Cd^{2+} levels. SA application prevented Cd^{2+}-induced electrolyte leakage (Table 2).

Effect of Cd^{2+} and SA on hydrogen peroxide (H_2O_2), lipid peroxidation (MDA) and antioxidant enzymes

The influence of Cd^{2+} and SA on H_2O_2, MDA and antioxidant enzymes is shown in Table 3. Hydrogen peroxide (H_2O_2) content showed approximately 88.0% and 156.5% rise at 0.75 and 1.50 mM Cd^{2+}, respectively. However, exogenously applied SA caused a substantial decrease of 15.6% and 22.0% at the same concentrations of Cd^{2+}, respectively. Lipid peroxidation was appraised in terms of MDA content. The MDA content was observed to be 25.7% at 0.75 mM Cd^{2+} and 74.3% at 1.50 mM Cd^{2+}. Lipid peroxidation reduced by 15.9% and 16.4% on foliar application of SA to 0.75 and 1.50 mM Cd^{2+}-treated plants, respectively. Superoxide dismutase (SOD) activity increased with the increase in exogenous Cd^{2+} level, and the increase was observed to be 18.7% and 30.9% at 0.75 and 1.50 mM Cd^{2+}, respectively. However, exogenous application of SA caused a significant decline in SOD activity in Cd^{2+}-treated plants. The decline was noticed to be 15.1% and 5.0% at the same concentrations of Cd^{2+}, respectively. Catalase (CAT) activity decreased under Cd^{2+} stress, being more marked (26.0%) at 1.50 mM Cd^{2+}. However, exogenous application of SA further decreased CAT activity (14.1%) in Cd^{2+}-treated pea plants. Ascorbate peroxidase (APX) activity was stimulated by Cd^{2+} toxicity as compared to that in the control. Maximum APX activity (20.0%) was observed at 1.50 mM Cd^{2+}. SA application reduced the activity of APX in Cd^{2+}-treated plants, being more marked (15.4%) at 0.75 mM Cd. Glutathione reductase (GR) also increased under Cd^{2+} stress. The magnitude of increase in GR activity was 17.0% at 1.50 mM Cd^{2+}. Exogenously applied SA reduced the levels of GR in all Cd^{2+}-treated plants.

Effect of Cd^{2+} and SA on macro- and micronutrients

Cadmium caused a marked reduction in shoot and root contents of N, P, K, Fe, Mn and Zn in the present study as shown in Table 4. For example, N content decreased with the increase in the concentration of Cd^{2+} (14.0% at 0.75 mM Cd^{2+} and 32.6% at 1.50 mM Cd^{2+} in shoots as well as 6.5% at 0.75 mM Cd^{2+} and 21.5% at 1.50 mM Cd^{2+} in roots), but foliar applied SA mitigated the negative effects of Cd^{2+} by restoring the N content in both shoots and roots. The contents of P and K decreased to 50.0% and 37.8% in shoots as well as 52.4% and 39.8% in roots, respectively at 1.50 mM Cd^{2+}. Exogenous application of SA helped the pea plants to restore these elements. Cadmium caused significant reductions in Fe, Mn and Zn contents in shoots and roots of pea plants under both Cd^{2+} treatments (0.75 and 1.50 mM). These reductions were more pronounced at 1.50 mM Cd^{2+}, however, a significant restoration in Fe, Mn and Zn contents were observed by foliar application of SA.

IV. Discussion

In the present study, exposure to high Cd^{2+} levels in the growth medium resulted in decreased shoot and root fresh and dry mass of pea plants. The growth inhibition was found to be associated with Cd^{2+}-induced decrease in the contents of photosynthetic pigments and nutrients as well as the relative water content. Growth inhibition by Cd^{2+} 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.,[36]. Similar to our study, Cd^{2+} application reduced fresh weights of shoots and roots close to 50%, and dry weights of shoots and roots by about 35% in barley (Metwally et al., [23], as well as reduced plant dry mass by about 54% in bean Rady,[37]. Attenuate effect of SA on the growth of pea plants under Cd^{2+} 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,[38], water relations Barkosky and Einhellig, [39], stomatal regulation (Arfan et al.,[40], photosynthetic capacity and growth (Popova et al.,[18], and antioxidant system Ahmad et al.,[41]. However, in the present investigation, exogenous application of SA improved shoot fresh weight of pea plants exposed to Cd^{2+} stress. This could have been due to the reason that Cd^{2+} is retained in roots and a very small amount of it is transported to shoots Caltado et al.,[42]. Choudhury and Panda [43] reported that Cd^{2+} content in SA non-primed rice roots was higher as compared to that in SA primed roots. The SA-induced differential accumulation of Cd^{2+} was considered as one of the potential physiological effects of SA on plants (Choudhury and Panda,[43]. SA is also known to reduce the accumulation of heavy metals other than Cd^{2+} Yang et al.,[44]. SA caused reduction in Cd^{2+} uptake in hemp (*Cannabis sativa*) and hence alleviated Cd toxicity Shi et al.,[45].

Hydrogen peroxide (H_2O_2) production increased markedly in pea plants upon Cd^{2+} treatment. Increase in H_2O_2 content is also reported in other plants upon Cd^{2+} treatment Kuo and Kao, [46].SA treatment decreased the level of H_2O_2 and led to reduce Cd^{2+}-induced oxidative injuries in pea plants in the present study. Similar results have also been recently reported by Ahmad et al. [41]that a decrease in H_2O_2 took place upon SA treatment in mustard seedlings exposed to Cd^{2+} stress. The SA-induced reduction in H_2O_2 content in Cd^{2+}-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^{2+} stress.

Accumulation of MDA, a product of lipid peroxidation, is commonly used as one of the potential indicators of oxidative stress (Skórzyńska-Polit and Krupa,[47]. Monteiro et al.[48] observed increased MDA content due to Cd^{2+} stress in lettuce. Furthermore, Zhang et al. [49]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^{2+}-induced enhancement in MDA content in the pea plants decreased due to SA application. These findings are parallel to what Ahmad et al. [41]observed that exogenous treatment of mustard seedlings with SA decreased the MDA content.

Cd^{2+}-induced oxidative stress leads to increased activities of antioxidative enzymes in plants Ahmad et al.,[41]. For example, an increase in the SOD, APX and GR activities was found after the application of Cd^{2+} in *Bacopa monnieri* Mishra et al., [15], *Triticum aestivum* Khan et al.,[50], *Brassica juncea* (Mobin and Khan, [51], and *phaseolus vulgaris* Rady,[37], whereas CAT activity was declined in *Bacopa monnieri* (Mishra et al., 2006), *Phragmites australis* (Iannelli et al., [52], and *phaseolus vulgaris* Rady,[37]. In the present study, Cd^{2+} 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^{2+}-treated plants. SA is an iron chelating molecule which can directly scavenge hydroxyl radicals (Dinis et al.,[53]. The Cd^{2+}-induced high levels of SA in the pea plants may have functioned directly as a prospective antioxidant to counteract the reactive oxygen species (ROS). In another study, Guo et al. [54]reported that exogenously applied SA improved rice Cd^{2+} tolerance by accelerating the activities of enzymes involved in the antioxidant defense system. Senaratna et al. [13]reported that SA can induce antioxidant activity under multiple stresses. Mba et al. [55]also observed that the activity of SOD in cabbage increases with the increase in external concentration of Cd^{2+}, but the SA treatment in the culture reduced the activity of SOD. Glutathione reductase (GR) is known to catalyze some vital steps of the ascorbate–glutathione cycle. 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,[56]. GR activity increased upon Cd^{2+} treatment in the present study, but it was suppressed by SA treatment. Similar results were observed by Metwally et al. [23]in barley seedlings.

In the present study, Cd^{2+} application caused a marked increase in intrinsic SA content in the leaves of pea plants. Similar Cd^{2+}-induced increase in SA has been found also in pea (Krantev et al.,[57[; Popova et al.,[18]. However, exogenous application of SA to Cd^{2+}-treated plants resulted in decreased accumulation of intrinsic SA in Cd^{2+}-treated pea 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^{2+}-treated pea plants and SA application further enhanced the proline levels in these plants. Enhanced proline accumulation in response to Cd^{2+} toxicity has been earlier demonstrated in *Triticum aestivum*, *Vigna radiate*, *Helianthus annuus* and *phaseolus vulgaris* Rady,[37]. Thus, proline accumulation is a potential indicator of stress tolerance (Ashraf and Foolad,[58].

Cd^{2+} application can lead to a deficiency of macro- and micronutrients in plants (Larbi et al.,[59]; Ramos et al., [60], which may cause other changes in plant metabolism. Cd^{2+} significantly disturbs ionic homeostasis, but SA can overcome this perturbance. For example, Cd^{2+}-induced decrease in K content may have been due to decreased K uptake caused by the antagonistic effect of Cd^{2+} Murphy et al.,[61]. Nitrogen, phosphorus, iron, manganese and zinc contents in leaves of pea plants decreased, but the exogenous application of SA attenuated the negative effect of Cd^{2+} on these nutrients.

V. Conclusion

SA-induced alleviation of the negative effects of Cd^{2+} toxicity may have been due to the following reasons. SA-treated pea plants accumulated considerably higher levels of Cd^{2+} 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^{2+} to upper plant parts i.e. shoots and hence promote shoot growth. SA also allayed the Cd^{2+}-induced oxidative damages. The values of MDA and H_2O_2 of SA treated-plants were considerably lower than those in the Cd^{2+} treated-plants. Thus, the adverse effects of Cd^{2+} toxicity on growth, enzymes involved in the oxidative defense mechanism and nutrient uptake in pea plants can be alleviated by foliar spray of SA.

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TABLE 1

Effect of Cd^{2+} (0.75 and 1.5 mM) and SA (2.0 mM) on shoot and root fresh and dry weights of pea plants.

Treatment	Parameter			
	Shoot FW (g plant^{-1})	Root FW (g plant^{-1})	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
0 Cd	15.9^{b}	7.1^{b}	8.1^{a}	3.9^{a}
0 Cd + SA	17.1^{a}	8.0^{a}	8.4^{a}	4.1^{a}
0.75 Cd	13.5^{d}	5.2^{d}	7.2^{c}	3.1^{c}
0.75 Cd + SA	14.5^{c}	5.9^{c}	7.8^{b}	3.6^{b}
1.50 Cd	10.2^{f}	3.8^{f}	5.5^{d}	2.4^{e}
1.50 Cd + SA	11.5^{e}	4.4^{e}	5.9^{d}	2.8^{d}

Means with different letters within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level (n = 6).

TABLE 2

Effect of Cd^{2+} (0.75 and 1.5 mM) and SA (2.0 mM) on leaf salicylic acid (mg kg^{-1} FW), shoot and root Cd (mg kg^{-1} DW), leaf chlorophyll and carotenoids (mg g^{-1} FW), leaf proline (mg kg^{-1} DW), relative water content (RWC) and electrolyte leakage (EL%) in pea plants.

Parameter	Treatment					
	0 Cd	0 Cd + SA	0.75 Cd	0.75 Cd + SA	1.50 Cd	1.50 Cd + SA
Salicylic acid	24.8^{e}	27.9^{e}	45.8^{c}	38.2^{d}	87.3^{a}	52.4^{b}
Cd (in shoot)	ND*	ND	6.71^{c}	4.54^{d}	10.93^{a}	8.41^{b}
Cd (in roots)	ND	ND	14.53^{b}	8.04^{c}	19.34^{a}	15.26^{b}
Total Chlorophyll	1.24^{a}	1.21^{a}	1.19^{a}	1.25^{a}	0.89^{b}	1.15^{a}
Carotenoids	0.32^{c}	0.39^{b}	0.24^{d}	0.33^{c}	0.40^{b}	0.44^{a}
Free proline	38.7^{e}	56.8^{d}	39.2^{e}	68.8^{c}	76.3^{b}	92.4^{a}
RWC %	76.8^{a}	70.4^{b}	67.2^{b}	74.9^{a}	48.7^{d}	56.6^{c}
EL%	8.4^{d}	9.8^{c}	11.3^{b}	10.1^{c}	14.3^{a}	11.8^{b}

*ND = not detectable

Means with different letters within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level (n = 6).

TABLE 3

Effect of Cd^{2+} (0.75 and 1.5 mM) and SA (2.0 mM) on hydrogen peroxide [H_2O_2 (µmol g^{-1} DW)], malondialdehyde [MDA (mmol g^{-1} FW)], superoxide dismutase [SOD (EU mg^{-1} protein)], catalase [CAT (EU mg^{-1} protein)], ascorbate peroxidase [APX (EU mg^{-1} protein)] and glutathione reductase [GR (EU mg^{-1} protein)] in pea plants.

Treatment	Parameter					
	H_2O_2	MDA	SOD	CAT	APX	GR
0 Cd	2.3^{d}	3.5^{d}	123^{c}	173^{a}	5.0^{b}	5.3^{b}
0 Cd + SA	2.2^{d}	3.7^{d}	129^{c}	160^{ab}	4.9^{b}	5.2^{bc}
0.75 Cd	4.5^{b}	4.4^{c}	146^{b}	149^{b}	5.2^{b}	5.7^{a}
0.75 Cd + SA	3.8^{c}	3.7^{d}	124^{c}	139^{c}	4.4^{c}	4.7^{c}
1.50 Cd	5.9^{a}	6.1^{a}	161^{a}	128^{c}	5.8^{a}	6.2^{a}
1.50 Cd + SA	4.6^{b}	5.1^{b}	153^{ab}	110^{d}	5.3^{b}	5.8^{a}

Means with different letters within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level (n = 6).

TABLE 4

Effect of Cd^{2+} (0.75 and 1.5 mM) and SA (2.0 mM) on macro- and micronutrients (mg g^{-1} DW) in shoots and roots of pea plants.

Parameter	Treatment					
	0 Cd	0 Cd + SA	0.75 Cd	0.75 Cd + SA	1.50 Cd	1.50 Cd + SA
Shoot N	2.36^{a}	2.23^{a}	2.03^{b}	2.31^{a}	1.59^{c}	2.04^{b}
Shoot P	0.48^{a}	0.49^{a}	0.36^{c}	0.43^{b}	0.24^{d}	0.37^{c}
Shoot K	2.67^{a}	2.61^{a}	2.36^{a}	2.59^{a}	1.66^{c}	2.03^{b}
Shoot Fe	0.31^{a}	0.29^{a}	0.21^{c}	0.24^{b}	0.14^{e}	0.18^{d}
Shoot Mn	0.20^{a}	0.21^{a}	0.16^{b}	0.21^{a}	0.10^{c}	0.16^{b}
Shoot Zn	0.08^{a}	0.06^{b}	0.04^{d}	0.06^{b}	0.02^{e}	0.05^{c}
Root N	2.14^{a}	2.08^{a}	2.00^{a}	2.16^{a}	1.68^{b}	1.96^{a}
Root P	0.63^{a}	0.61^{ab}	0.54^{c}	0.59^{b}	0.30^{e}	0.48^{d}
Root K	2.94^{a}	2.86^{a}	2.59^{bc}	2.81^{ab}	1.77^{d}	2.37^{c}
Root Fe	0.36^{a}	0.37^{a}	0.28^{c}	0.31^{b}	0.18^{e}	0.25^{d}
Root Mn	0.28^{a}	0.26^{a}	0.23^{b}	0.26^{a}	0.15^{c}	0.22^{b}
Root Zn	0.11^{a}	0.11^{a}	0.08^{bc}	0.09^{b}	0.05^{d}	0.07^{c}

Means with different letters within each attribute were separated by Duncan's Multiple Range Test (DMRT) at 5% level (n = 6).