Journal of Plant Sciences
2014; 2(6): 276-281
Published online December 05, 2014 (http://www.sciencepublishinggroup.com/j/jps)
doi: 10.11648/j.jps.20140206.13
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Salicylic acid-mediated alleviation of cadmium toxicity in maize leaves

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To cite this article:
Khalid Al-Mureish, Nasim Abdual Rahim Mohammed Othman, Abdulbasset Mohammed Ahmed Al-Hakimi. Salicylic Acid-Mediated Alleviation of Cadmium Toxicity in Maize Leaves. *Journal of Plant Sciences.* Vol. 2, No. 6, 2014, pp. 276-281.
doi: 10.11648/j.jps.20140206.13

Abstract: The present study was carried out to investigate the effects of salicylic acid (SA) pretreatment on the activities of antioxidant enzymes and some biochemical attributes in maize (*Zea mays* L.) seedling leaves exposed to cadmium (Cd) stress. The Cd toxicity in maize leaves was revealed by reduction of ascorbate and cysteine concentrations. However, a remarkable increase of such non-enzymatic antioxidants concentrations was noticed on the pretreatment with SA. Cadmium- induced oxidative stress also showed a pronounced increase in hydrogen peroxide (H_2O_2), lipid peroxidation, electrolyte leakage (El) and proline production. The important point to be emphasized here is that the pretreatment with SA attenuated the adverse effects of Cd on these attributes. Cadmium-induced activities of some key antioxidant enzymes, peroxidase (POD) and ascorbate peroxidase (APX) was further increased on the exposure to SA. While the lower catalase (CAT) activity dues to Cd toxicity was increased by SA pretreatment.

Keywords: Antioxidants, Cadmium, Hydrogen Peroxide, Lipid Peroxidation, Salicylic Acid, *Zea mays* L.

1. Introduction

Maize (*Zea mays* L.) is one of the important cereals grown in most countries in the world with total areas exceeding 160 million ha. Maize producers want to buy quality seeds, because of it profound influence on the yield of maize [1].

Cadmium is a highly toxic trace element that enters the environment mainly from industrial processes and phosphate fertilizers and negatively affects plant growth and development. High Cd levels in plants can cause oxidative stress by producing reactive oxygen species (ROS) [2] that react with lipids, proteins, pigments and nucleic acid and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus resulting toxic effects. To minimize and / or to protect against the toxic effects of these damaging ROS, plants evolve highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction in order to maintain cellular redox homeostasis. Plants utilize enzymes like APX, POD and CAT as well as low molecular weight antioxidants like ascorbate (ASA) and glutathione (GSH) to scavenge ROS [3].

Interestingly, SA acts as a potential non-enzymatic antioxidant and a plant growth regulator, which influences a range of diverse physiological processes and promotes the plant resistance to biotic and abiotic stresses [4]. Recently, it has been reported that exogenous SA ameliorates the damaging effects of heavy metals in maize [5], drought stress in soybean [6] and salt stress in wheat [7].

The present work was devoted to show the changes of the antioxidant system in response to Cd toxicity and the effect of SA pretreatment on maize leaves. The antioxidant status was monitored through analyzing the enzymatic and non-enzymatic antioxidants and determining the lipid peroxidation, H_2O_2 levels, electrolyte leakage and proline content. In addition, this work provided clear evidences for SA protective interference action and regulation of oxidative stress caused by Cd toxicity in maize seedling leaves.

2. Materials and Methods

2.1. Plant Materials and Treatments

Maize seeds (*Zea mays* L.) were surface sterilized with 5% sodium hypochlorite solution for 5 minutes and washed thoroughly with distilled water. The seeds were then divided into two halves. One half of the seeds were soaked in 500 µM

SA solution for 6 hours, while the other half was soaked in water. Both groups were then allowed to germinate on moist filler paper in the dark. Three–day-old, dark-grown seedlings were transplanted to a polyethylene post containing a continuously aerated full strength Hoagland's nutrient solution. The nutrient solution was aerated twice a day, and changed three times a week. $CdCl_2$ was added into the nutrient solution at 50 and 100 µM. The plants grew under controlled environmental conditions. Five replicates were maintained for each treatment concentration. After 14 days of growth, i.e. 3 days after soaking the plants were harvested for analyses.

2.2. Determination of Hydrogen Peroxide

The hydrogen peroxide content was determined according to [8]. A 500 mg leaf tissue was homogenized with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 12,000 rpm for 15 minutes. A 0.5- ml supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide (KI) solution. The absorbance of the supernatant was measured at a wavelength of 390 nm.

2.3. Determination of Lipid Peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to the method described in [9]. Leaves (500 mg) were homogenized with 2.5 ml of 0.1 % TCA solution. The extract was centrifuged at 10,000 rpm for 10 minutes. A 4- ml 20 %TCA solution containing 0.5 % thiobarbituric acid (TBA) was added to every 1ml of the aliquot. After properly treating the mixture, it was centrifuged at 10,000 rpm for 15 minutes, and the absorbance of the supernatant was measured at a wavelength of 532 nm. The level of lipid peroxidation was expressed as µmol of MAD formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.4. Determination of Electrolyte Leakage

Electrolyte leakage is used to assess the membrane permeability. Twenty leaf discs were put in a boiling tube containing 10 ml deionized water and electrical conductivity was measured (EC_o). The contents were healed at 50 and 60 ºC for 25 minutes in a water bath and EC was measured (EC_1). Later, the contents were boiled for 10 minutes and the EC was again recorded (EC_2).The electrolyte leakage was calculated using the formula:

$$\text{Electrolyte leakage (\%)} = (EC_1 - EC_o) / (EC_2 - EC_o) \times 100.$$

2.5. Assay of Enzymatic Antioxidants

Catalase activity was measured by monitoring the destruction of H_2O_2 [10]. The reaction mixture consisted of 200 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2, 0.05 ml enzyme extract. The decrease in absorbance due to the decomposition of H_2O_2 was recorded at a wavelength of 240 nm. The activity was calculated using the extinction coefficient (ε 40 mM^{-1} cm^{-1}) and expressed in µmole of H_2O_2 min^{-1} mg^{-1} protein. The POD activity was determined based on guaiacol oxidation [11]. The reaction mixture contained of 50 mM guaiacol, 10 mM H_2O_2 and 0.05 ml enzyme extract. The increase in absorbance due to formation of tetraguaiacol was monitored at 470 nm. The activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} and expressed as µmol of H_2O_2 min^{-1} mg^{-1} protein. The APX activity was determined according to [12]. APX was assayed in 3 ml reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.5 mM H_2O_2, the reaction was started with addition H_2O_2. APX activity was calculated as the decrease in A_{290nm} of ascorbate using an extinction of 2.8 mM^{-1} cm^{-1}. APX activity was expressed as µmol of H_2O_2 min^{-1} mg^{-1} protein, taking into consideration that 1.0 mol of ascorbate is required for the reduction of 1.0 mol of H_2O_2.

2.6. Determination of Soluble Protein

To estimate soluble proteins, leaf samples (100 mg) were boiled in 10 ml distilled water for 2 hours. After cooling, the water extract was centrifuged at 6000 rpm, the supernatant was decanted and made up to a definite volume by distilled water and the soluble proteins were determined according to [13].

2.7. Assay of Ascorbate Concentrations

Ascorbate was estimated according to [14]. Leaf tissues (100 mg) were homogenized in 5% (w/v) sulfosalicylic acid, and were then centrifuged at 10,000 rpm for 10 minutes. The reaction mixture for ascorbate consisted of 2 ml 2% sodium molybdate, 2 ml 0.15M H_2SO_4, 1 ml 1.5 mM $NaHPO_4$ and 1 ml tissue extract. It was incubated in water bath at 60 ºC for 40 minutes, cooled and centrifuged at 3000 rpm for 10 minutes, and the absorbance was measured at 660 nm. A standard curve with ascorbate was used and calculated as µg mg^{-1} of fresh leaf weight.

2.8. Assay of Cysteine Concentrations

Cysteine was estimated as described in [15]. Leaf tissues (100 mg) were homogenized in 5% chilled perchloric acid and centrifuged at 10,000 rpm for 10 minutes at 4 ºC. Cysteine content was measured in supernatant using acid ninhydrin reagent at 560 nm. A standard curve with cysteine was used and calculated as µg mg^{-1} of fresh leaf weight.

2.9. Determination of Proline

Proline concentration in the leaves was determined spectrophotometerically as described in [16]. 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 minutes was mixed with an equal volume of acetic acid and acid ninhydrin and incubated for 1hour at 100 ºC. The chromatophore containing toluene was then aspirated from the aqueous phase, and its absorbance read spectrophotometrically at 520 nm using toluene as a blank. Proline content was expressed in µM g^{-1} of fresh leaf weight.

2.10. Statistical Analysis

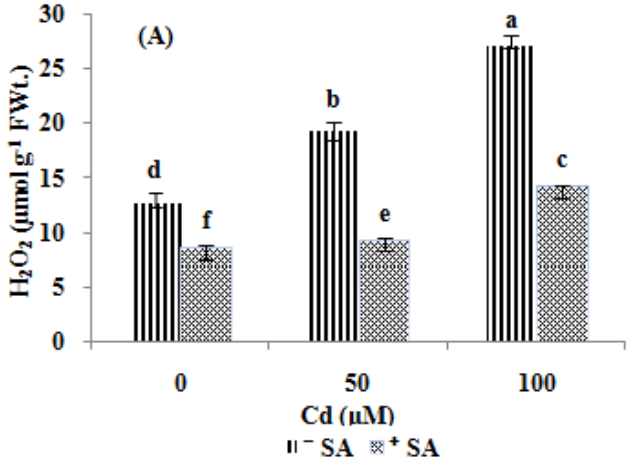
Data were statistically analyzed in experimental observations wherever required, and the results were expressed as meant ±SD of five independent replicates of each independent experiment. The significance of differences between control and each treatment was analyzed using analysis of variance at $P < 0.05$ level of significance.

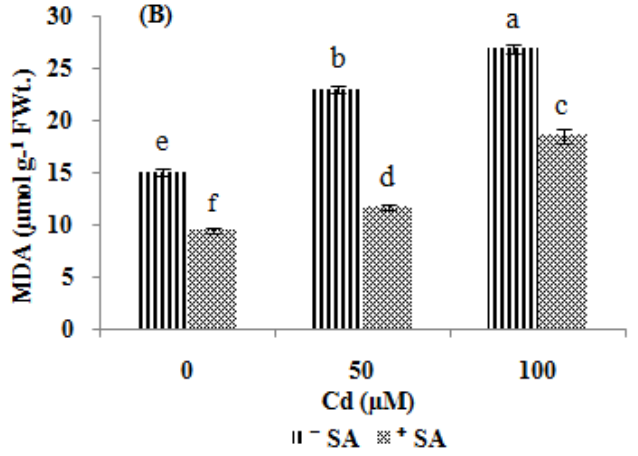
3. Results

Hydrogen peroxide content in maize leaves showed approximately 52.3% and 114.7% rise at 50 and 100 µM Cd, respectively (Fig. 1A). However, exogenously applied SA caused a substantial decrease of 57.5% and 47.7% at the same concentrations of Cd (Fig. 1A). Pretreatment of SA alone also decreased H_2O_2 content by 32.2 % compared with control (Fig. 1A).

Malondialdehyde (MDA) level was used as an indicator for lipid peroxidation and represents a balance of oxidative stress that induced production of MDA in relation to Cd treatments. Thus, MDA can be regarded as a sink for oxidative radicals. However, the results given in Fig. 1B showed a remarkable increase in MDA level as a result of Cd treatment. This is an indicative of the enhanced lipid peroxidation degree with increasing Cd concentration. The maximum increase in MDA was 52.6% and 78.5% at 50 and 100 µM Cd, respectively. SA treatment significantly decreased MDA level compared with Cd treated levels (Fig. 1B). The maximum decrease in MDA by SA treatment reached 48.9% at 50 µM Cd and 30.8% at 100 µM Cd. Alone treatment with SA as well significantly decreased MDA level by 37.0%.

Electrolyte leakage was increased with increasing Cd concentration. This increase was approximately estimated to be 66.4 % and 79.3 % at 50 and 100 µM Cd, respectively compared to the control (Fig. 1C). Generally, SA pretreatment thus showed antagonizing partially or completely for the stimulatory effects of Cd toxicity on electrolyte leakage (Fig. 1C).





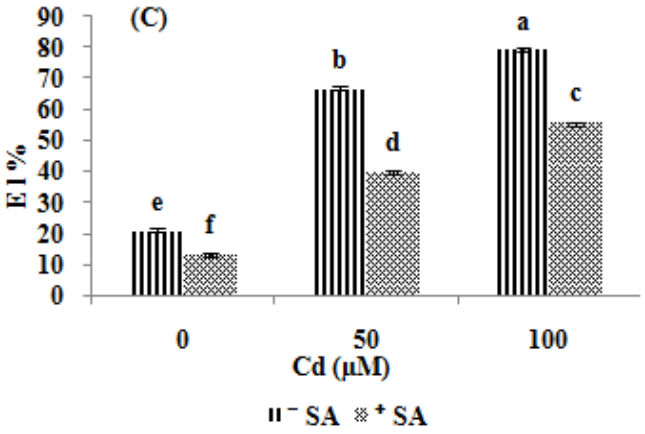


Figure 1. *The action of SA treatment in ameliorating the adverse effects of Cd toxicity on (A) H_2O_2, (B) MDA and (C) EI in leaves of maize seedlings. Values shown are the mean ± SD (n= 5). Different litters indicate significantly different values at $p < 0.05$.*

Exposure of Cd decreased CAT activity in leaves maize by approximately 53.2% and 63.6% at 50 and 100 µM Cd, respectively compared with control (Fig. 2A). SA pretreatment alone also decreased CAT activity by 43.5%. However, SA pretreatment significantly increased CAT activity in Cd-stressed maize leaves compared with that of Cd alone treatment (Fig. 2A). The activity of CAT increased by 76.6 % and 102.2% at 50 and 100 µM Cd, respectively.

A significant increase in POD activity was observed in response to Cd stress (Fig. 2B). Cd- stressed leaves showed approximately 48.1% and 189.6% rise at 50 and 100 µM Cd, respectively. SA pretreatment initially increased POD activity in leaves maize by 31.5 % and 17.0 % at 50 and 100 µM Cd, respectively. SA alone also increased POD activity by 48.1%.

Significant increase in the APX activity was noticed in response to Cd application (Fig. 2C). For instance, doses of 50 and 100 µM Cd increased the APX activity by 25.3% and 55.2%, respectively. Application of SA also increased the APX activity by 15.3% and 27.0% at 50 and 100 µM Cd, respectively (Fig. 2C). SA alone significantly increased APX activity.

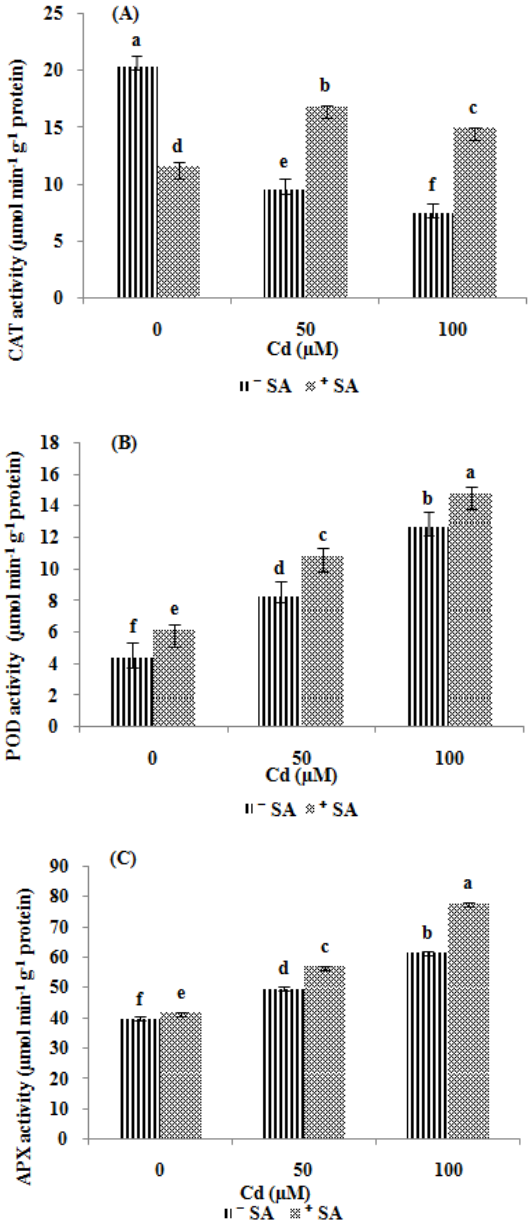


Figure 2. *The action of S) treatment in ameliorating the adverse effects of Cd toxicity on (A) CAT, (B) POD and (C) APX activity in leaves of maize seedlings. Values shown are the mean ± SD (n= 5). Different litters indicate significantly different values at p < 0.05.*

Ascorbate content was markedly decreased by Cd stress. The decrease in ascorbate induced by Cd treatment was found to be 36.5% and 52.4% at 50 and 100 µM Cd, respectively. The adverse effects of Cd treatment on ascorbate content in leaves maize was partially by soaking seeds in SA (Fig. 3A). The ameliorative effects of SA on Cd induced reduction in ascorbate content were 49.8% and 43.0% at 50 and 100 µM Cd, respectively. SA alone significantly decreased ascorbate content by 17.2%.

The addition of Cd consistently decreased cysteine content in leaves maize in all treatments (Fig. 3B). The maximum reduction in cysteine content was 23.1% and 42.5% of control at 50 and 100 µM Cd, respectively. Pretreatment with SA initially increased cysteine content in leaves maize by 104.9% and 57.6% prior to 50 and 100 µM Cd treatment (Fig. 3B). SA alone significantly increased cysteine content by 61.0%.

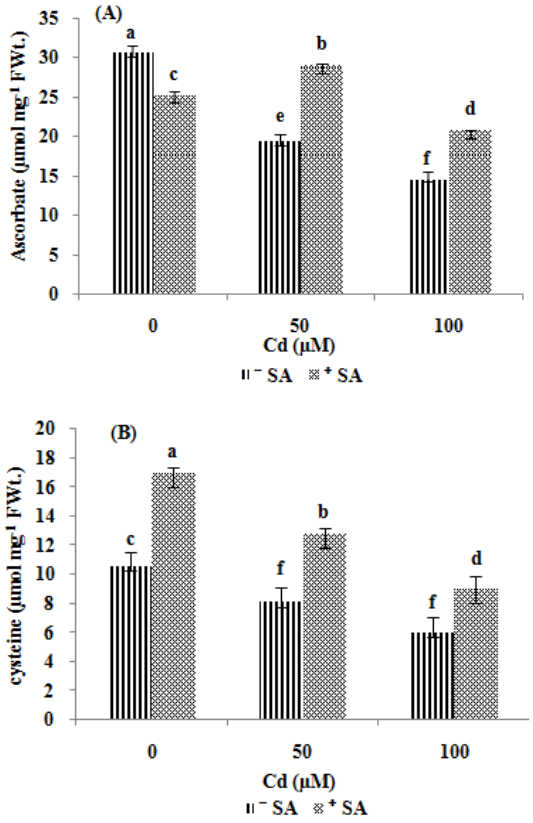


Figure 3. *The action of SA treatment in ameliorating the adverse effects of Cd toxicity on (A) ascorbate and (B) cysteine content in leaves of maize seedlings. Values shown are the mean ± SD (n= 5). Different litters indicate significantly different values at p < 0.05.*

Cd exposure increased proline content in leaves maize by 38.5% at 50 µM and 85.0% at 100 µM Cd compared with control (Fig. 4). Under Cd toxicity, SA pretreatment significantly decreased proline content compared with corresponding Cd alone treatment (by 42.1% and 50.0% at 50 and 100 µM Cd, respectively (Fig. 4). SA alone also reduced the proline content by 24.4%.

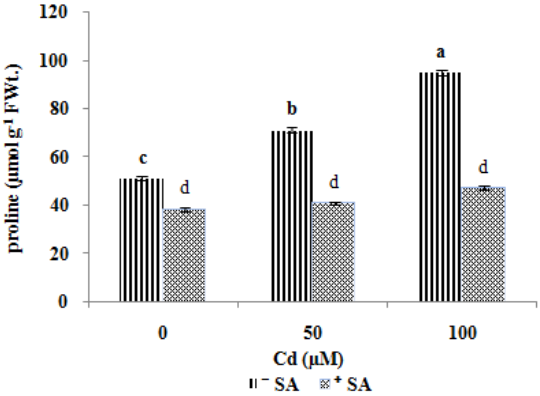


Figure 4. *The action of SA treatment in ameliorating the adverse effects of Cd toxicity on proline content in leaves of maize seedlings. Values shown are the mean ± SD (n= 5). Different litters indicate significantly different values at p < 0.05.*

4. Discussion

Hydrogen peroxide plays significant signals for the induction and regulation of stress enzymes such as APX [17]. In the present study, the exposure to Cd levels in the growth medium resulted in the increase of H_2O_2 level in maize leaves. It has been found that the increase of H_2O_2 production in wheat plants is associated with the activation of the ROS-producing enzymes and the inactivation of antioxidant enzymes [18]. The enhanced H_2O_2 levels detected in Cd-treated leaves should be indirectly originated from a decreased H_2O_2-scavenging rate and / or an increased H_2O_2 production by cell enzymatic and non-enzymatic processes. In any case, the production of H_2O_2 may be involved in the integration of cellular processes and in the adaptation to environmental stimuli [19], since, for instance, H_2O_2 is required for cross-linking cell wall components and for regulating gene expression associated with antioxidant defense [20]. In general, the applied SA has partially or completely antagonizing for the stimulatory effect of Cd toxicity on H_2O_2 level. The lowering in H_2O_2 levels under SA pretreatment is without doubt attributed to the increased activities of CAT and APX as a response to SA exposure.

Cadmium is not a redox active metal; nevertheless, it can indirectly generate ROS that can cause oxidative damage to cellular constituents including lipids. MDA is one of the end products which are produced as a result of lipid production damage by free radicals. Lipid production level as indicated by accumulated MDA. MDA increases significantly under Cd stress. This suggests that the Cd stress induced membrane injury, which may be due to changes in the membrane lipids or protein or both [21, 22, 23]. The applied SA has partially or completely antagonizing for the stimulatory effect of Cd stress on MDA level of maize leaves. However, SA decreases the content of lipid peroxidation by inhibiting production of hydroxyl radical [22]. The beneficial effect of SA could be attributed to modified compartmentalization, and increased activities of defense mechanisms such as antioxidant enzymes which could be involved in lowering Cd toxicity [24].

Like MDA, the electrolyte leakage also increased significantly under both two Cd concentrations. This confirmed that Cd toxicity in leaf maize was linked to free radical processes in membrane components leading to alterations in membrane stability and increasing their permeability [21, 23]. The applied SA was generally effective in antagonizing partially or completely for the stimulatory effect of Cd stress on electrolyte leakage. The remarkable decrease in electrolyte leakage is a clear evidence for the reduction of membrane damage, increased membrane stability and high tolerance of plants under SA pretreatment [5, 22]. In one hand, CAT activity markedly decreased in maize seedling leaves with Cd toxicity. The decrease in CAT activity may be attributed to the degradation caused by peroxisomal proteases as well as the photoinactivation of enzymes [25, 26]. It can be noted that the pretreatment with SA alleviated the inhibitory effects of Cd on CAT activity [5]. In other hand, APX activity was remarked increased with increasing Cd concentration compared with the control. This increase in APX activity in Cd-treated plants seems to be in response of increased accumulation of H_2O_2 [26]. The SA pretreatment was found to continuously increase the APX activity in Cd-stressed maize leaves. This result is in a good agreement with our previous study [21, 22]. It is worthwhile to note that the dissimilar CAT and APX activities exhibited under both Cd stress and SA treatment may be due to the competition between APX and CAT on the same working substrate, H_2O_2, which shows higher activity with APX. This could be explained by the fact that if the detoxification of H_2O_2 is mainly occurred by APX, the CAT activity would be declined due to the lesser availability of H_2O_2 [26].

POD activity increase continuously with increasing Cd concentration. In contrast to what have been reported in sorghum leaves under Cd toxicity [22]. This increase in POD activity after Cd treatment indicates that H_2O_2 may further be produced by POD through oxidation of various molecules, such as NAD(P)H [23]. Moreover, the pretreatment with SA significantly enhanced POD activity in leaves of maize seedlings with or without Cd treatment.

It can also be noted that the treatment with Cd decreases ascorbate content in leaves of maize seedlings and thereby reducing H_2O_2 detoxification [27] and plant defense against oxidative stress [2]. The pretreatment with SA also significantly increased the ascorbate content. This is well agreed with our results reported earlier [28].

Cysteine content was significantly declined under Cd treatment. This may inhibit the synthesis of glutathione which acts as a putative ligand for metals in plant cells [28]. Generally, the adverse effects of Cd stress on cysteine content in leaves maize was partially or completely alleviated by soaking seeds in SA.

Proline content increased markedly in the Cd-treated plants. Enhanced proline accumulation in response to Cd toxicity has been earlier demonstrated in sorghum [22]. However, the pretreatment with SA before exposure to Cd decreased the stimulatory effect of Cd on proline accumulation. This noticeable decrease in proline content in wheat plants grown from SA-pretreated seeds indicates a particle recovery from Cd stress [29].

5. Conclusion

SA-induced alleviation of the negative effects of Cd toxicity may be attributed to the following reasons:

(i) SA allayed the Cd-induced oxidative damages.
(ii) The lowering levels of H_2O_2, MDA, electrolyte leakage and proline content of SA-pretreated plants in comparison with Cd exposed plants.
(iii) SA- altered activities of CAT, POD and APX plants treated with and without Cd.

It is interesting to conclude that the adverse effects of Cd toxicity in enzymatic antioxidants involved in the oxidative defense mechanism in plants can be significantly alleviated by application of SA.

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