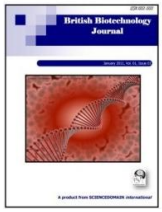

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Effects of Exogenous Salicylic Acid on the Antioxidative System in Bean Seedling Treated with Manganese

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Authors' contributions

This work was carried out in collaboration between all authors. Author IS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NY, WD and YC managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

In the present study we investigated the role of salicylic acid (SA) in regulating Mn-induced oxidative stress in bean (*Phaseolus vulgaris)* leaves. Exposure of plants to 100 µM Mn inhibited biomass production and intensively increased Mn accumulation in leaves. Concomitantly, Mn significantly enhanced protein carbonyl, H_2O_2 content and lipid peroxidation as indicated by malondialdehyde (MDA) accumulation. SA (10, 50 and 100 µM) pretreatment alleviated the negative effect of Mn on plan growth and led to decrease in oxidative stress induced by Mn stress. Furthermore, SA enhanced the activities of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), but lowered that of superoxide dismutase (SOD, EC 1.15.1.1) and guaiacol peroxidase (POD, EC

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1.11.1.7). The data suggest that the beneficial effect of SA could be related to avoidance of oxidative damage upon exposure to Mn thus reducing the negative consequences of oxidative stress caused by Mn toxicity.

Keywords: Manganese; salicylic acid; oxidative stress; antioxidants; Phaseolus vulgaris.

1. INTRODUCTION

Manganese (Mn) is a crucial trace metal required in numerous cellular processes, including metabolism and oxidative stress defense [1]. However, it has been reported that excess Mn disturbs the metabolism of plants and inhibit the plant growth [2]. Mn toxicity symptoms in plants appear first in shoots and these are often more sensitive toxicity parameters than vegetative growth [3]. For many species such as barley [4], bean [5], sunflower [6] and cow pea [7] first Mn toxicity symptoms are dark brown speckles, desiccation and shedding of old leaves [8]. The toxic effects of manganese can be related to the generation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet-}$) and hydrogen peroxide (H_2O_2) in the plants, which damage the membranes and all the macromolecules like lipids, proteins and nucleic acids in the cells [2].

Salicylic acid (SA), a naturally occurring plant hormone, has been shown to be an essential signal molecule involved in local defense reactions and induction of systemic resistance response of plants after pathogen attack [9]. The role of SA in plant tolerance to abiotic stresses such as drought [10], chilling [11], heat [12] and osmotic stress [13] has also been reported.

Salicylic acid (SA) is involved in regulating plant responses under heavy metal-induced toxicity [14-16]. Interestingly, the majority of SA-regulated heavy metal stresses in plants are involved in antioxidative responses, suggesting that SA is an internal signal molecule interacts with ROS signal pathway. In addition, SA could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzyme activity [17] and induction of the alternative respiratory pathway [18].

Bean (*Phaseolus vulgaris*) is a widely used plant cultured throughout the word. It accumulates considerable amount of some metals, such as Cd, Cu and Zn [19]. Since Mn produces oxidative damage in higher plants, an enhancement of the antioxidant properties of bean seedling is expected as a consequence of SA supplementation under Mn stress conditions. Therefore the present study was undertaken to evaluate the toxic mechanism associated with Mn exposure and to investigate the possible mediatory role of SA in protecting plants from Mn-induced oxidative stress.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Bean seeds (*Phaseolus vulgaris*) were disinfected with 1% NaOCl for 5 min, then washed thoroughly with distilled water and germinated between wet paper towels at 24ºC in the dark. Four days later, obtained seedlings were transferred into plastic beakers (6 L capacity, 6 plants per beaker) filled with nutrient solution containing: 1.0 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4, 2.0 mM KNO_3, 2.0 mM NH_4Cl, 50 µM EDTA–Fe–K, 30 µM H_3BO_3, 1.0 µM $ZnSO_4$, 1.0 µM $CuSO_4$ and 30 µM $(NH4)_6Mo_7O_{24}$. After an initial growth period of 7 days in different SA concentrations (10, 50 and 100 µM), treatments were performed by adding 100 µM $MnCl_2$ to the nutrient solution. Mn dose used in this work are chosen appropriately to expose the plants from low to moderate levels of Mn. Plants were grown in a growth chamber with a 16-h-day (25ºC)/8-h-night (20ºC) cycle, an irradiance of 150 µmol m^{-2} s^{-1}, and 65-75% relative humidity. The nutrient solution was buffered to pH 5.5 with HCl/KOH, aerated, and changed twice per week. After 4 days of Mn-treatment, roots of the harvested plantlets were soaked in 20 mM EDTA for 15 min to remove adsorbed metals and washed carefully using distilled water to eliminate any contamination. Primary leaves were harvested and immediately stored in nitrogen liquid. Three independent repetitions of the whole experiment were performed in order to check reproducibility. For biomass production, Mn determination and biochemical analyzes five plantlets from each replication of all treatments were selected.

2.2 Determination of Ion Concentrations

Dry plant material was powdered and wet-digested in acid mixture (HNO_3:$HClO_4$, 3:1, v/v)

at 100ºC. Mn concentrations were estimated by atomic absorption spectrometry (Perkin-Elmer, Analyst 300) using an air–acetylene flame.

2.3 Determination of Lipid Peroxidation, Hydrogen Peroxide and Protein Carbonyl Contents

The level of lipid peroxidation in plant leaves was determined by estimation of the thiobarbituric acid (TBA) reactive substances which was expressed as the malondialdehyde (MDA) concentration based on the method of Hodges et al. [19]. Lipid peroxidation level was expressed as nmol MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) levels were determined according to Sergiev et al. [20]. Results were expressed as nmol g^{-1} FW.

Protein carbonyls were determined using 2,4-dinitrophenylhydrazine (DNPH) and the basis of the assay involved the reaction between protein carbonyl and DNPH to form protein hydrazone [21]. Results were expressed as nmol DNPH conjugated mg^{-1} protein.

2.4 Determination of Antioxidative Enzyme Activities

Frozen leaf tissue (0.4 g) was homogenized in 4 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0, 0.4% PVPP) using a pre-chilled mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged for 30 min at 14,000 *g* at 4ºC. The supernatant was used for assays of the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and Peroxidase (POD). All spectrophotometric analyses were conducted at 25ºC.

The activity of SOD (EC1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Beauchamp and Fridovich [22]. SOD activity was expressed as U mg^{-1} protein.

CAT (EC1.11.1.6) activity was assayed by the decomposition of hydrogen peroxide according to Aebi [23]. CAT activity was expressed as U mg^{-1} protein.

APX (EC1.11.1.1) activity was determined by the method of Nakano and Asada [24]. APX activity was expressed as U mg^{-1} protein.

The activity of POD (EC1.11.1.7) was determined in terms of oxidation of guaiacol by measuring increase in absorbance at 470 nm [25]. POD activity was expressed as U mg^{-1} protein.

2.5 Determination of Soluble Protein Concentration

Soluble protein concentration was measured according to Bradford [26] using the bovine serum albumin (BSA) protein assay reagent (Pierce, BSA Protein Assay Kit, USA) with BSA as the standard protein. All spectrophotometric measurements were performed by using a Perkin Elmer's LAMBDA 25/35/45 UV/Vis spectrophotometer.

2.6 Statistical Analysis

All statistical analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by 1-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons with statistical significance of $P<0.05$. Number of replications (n) in tables/figures denotes individual plants measured for each parameter. Results were expressed as mean ± standard error of the mean (mean ± SEM).

3. RESULTS

3.1 Effects of SA Pretreatment on Seedling Growth under Mn Stress

Plant growth was negatively affected by Mn treatment (100 µM), reducing root and leaf dry matter by about 52.55% and 57.89% as compared with untreated plants (Table 1). Although SA pretreatments did not affect root dry matter of plants grown with or without Mn, they markedly alleviated Mn-induced leaf growth inhibition. Under Mn stress conditions, SA pretreatments enhanced leaf dry matter and the most significant effect was observed at 100 µM SA (increase by about 43% in leaf compared to Mn-treated plant) (Table. 1).

Table 1. Effects of SA pretreatment on root and leaf dry matter in 14-day-old bean plant submitted during 4 days to 100 µM $MnCl_2$. Data are means ± SEM (n=5). Values within rows followed by the same letter(s) are not significantly different according to Tukey's test, (P<0.05)

SA (µM)	Mn in nutrient solution	
	0 (µM)	100 (µM)
Root DM (g plant^{-1})		
0	0.137±0.009a	0.065±0.003b
10	0.147±0.011a	0.063±0.001b
50	0.140±0.017a	0.072±0.012b
100	0.142±0.012a	0.076±0.014b
Leaf DM (g plant^{-1})		
0	0.19±0.001b	0.08±0.004d
10	0.21±0.004b	0.11±0.002c
50	0.24±0.009a	0.12±0.001c
100	0.26±0.011a	0.14±0.002c

3.2 Effects of SA Pretreatment on Mn Distribution

Mn addition to the nutrient solution resulted in a high accumulation of this metal within plant organs reaching 3.14 mg g^{-1} DM in roots and a value of 4.55 mg g^{-1} DM in leaves (Table. 2). Pretreatment with SA before application of Mn markedly decreased Mn concentration in both roots and leaves. In comparison with Mn treatment, pretreatment with SA (100 µM) decreased Mn concentration by 19.11% and 29.45%, respectively in roots and leaves (Table 2).

Table 2. Effects of SA pretreatment on Mn distribution in 14-day-old bean plant submitted during 4 days to 100 µM $MnCl_2$. Data are means ± SEM (n=5). Values within rows followed by the same letter(s) are not significantly different according to Tukey's test, (P<0.05)

SA (µM)	Mn concentration (mg g^{-1} DM)	
	Roots	Leaves
0	3.14±0.11a	4.55±0.101a
10	2.95±0.14a	4.22±0.164a
50	2.82±0.16b	4.02±0.102b
100	2.54±0.08c	3.21±0.168c

3.3 Effects of SA Pretreatment on H_2O_2, MDA Concentration and Protein Carbonyl Contents in Leaves of Bean Seedlings under Mn Stress

Mn addition increased H_2O_2 concentration by more than twice as compared to the control (Table 3). SA alone did not significantly affect H_2O_2 production rate (Table 3). By contrast, its concentration in leaves of seedlings supplied with 10, 50 and 100 µM SA was decreased by respectively 16.59%, 21.99% and 30.54% relative to Mn-stressed plants grown without SA application.

As compared to the control, Mn-treated plants exhibited a higher leaf MDA concentration (Table 3). Plant pretreatment with SA before Mn application significantly decreased MDA level in a dose-dependent manner, the effect being more pronounced with the highest applied SA (100 µM) concentrations. However, no major changes were observed in MDA level in the presence of SA alone.

Protein carbonyl (PCO) content increased upon Mn exposure by approximately 33.45% as compared to the control (Table 3). By contrast, pretreatment with SA before Mn application decreased the level of PCO by about, 12, 15.8 and 21.6% at 10, 50 and 100 µM, respectively as compared to Mn-stressed plants (Table. 3).

3.4 Effects of SA Pretreatment on Antioxidant Enzyme Activities in Leaves of Bean Seedlings under Mn Stress

Under Mn stress conditions SOD and POD activities in leaves exposed to 100 µM Mn were observed to be 41.5-38% higher than those of the control (Figs. 1A-B). By contrast, CAT and APX activities were decreased in Mn-treated leaves by about 43.5% and 40% as compared to the control (Fig. 1C-D). SA pretreatment resulted in significant decreases in SOD and POD activities upon Mn exposure and alleviated the inhibitory effect Mn on CAT and APX activities. Leaf SOD and POD activities were strongly affected by SA pretreatment, especially at 100 µM (approximately, 24% and 20% lesser than Mn alone treatment). Moreover, SA were

effective in alleviating Mn-inhibited leaf APX and CAT activities, The most prominent effect was at 100 µM SA, the concentration that induced an increase of 26-30% in CAT and APX activities (Figs. 1C-D). However, no noticeable variation was observed in antioxidant enzyme activities of plants treated with SA only.

Table 3. Effects of SA pretreatment on MDA, H_2O_2 and PCO concentration in 14-day-old bean plant submitted during 4 days to 100 µM $MnCl_2$. Data are means ± SEM (n=5). Values within rows followed by the same letter(s) are not significantly different according to Tukey's test, (P<0.05)

Treatment		H_2O_2 (nmol g^{-1}FW)	MDA (nmol g^{-1}FW)	PCO (nmol DNPH mg^{-1} prot)
SA (µM)	Mn (µM)			
0	0	8.11±0.110c	5.80±0.150d	2.81±0.19c
10	0	7.48±0.019c	5.98±0.065d	2.60±0.11c
50	0	8.57±0.025c	5.02±0.144d	2.77±0.21c
100	0	8.99±0.054c	5.68±0.012d	2.86±0.14c
0	100	17.78±0.148a	11.43±0.11a	3.75±0.15a
10	100	14.83±0.108b	9.13±0.181b	3.30±0.15b
50	100	13.87±0.108b	8.24±0.101b	3.16±0.10b
100	100	12.35±0.108b	7.82±0.011c	2.94±0.21b

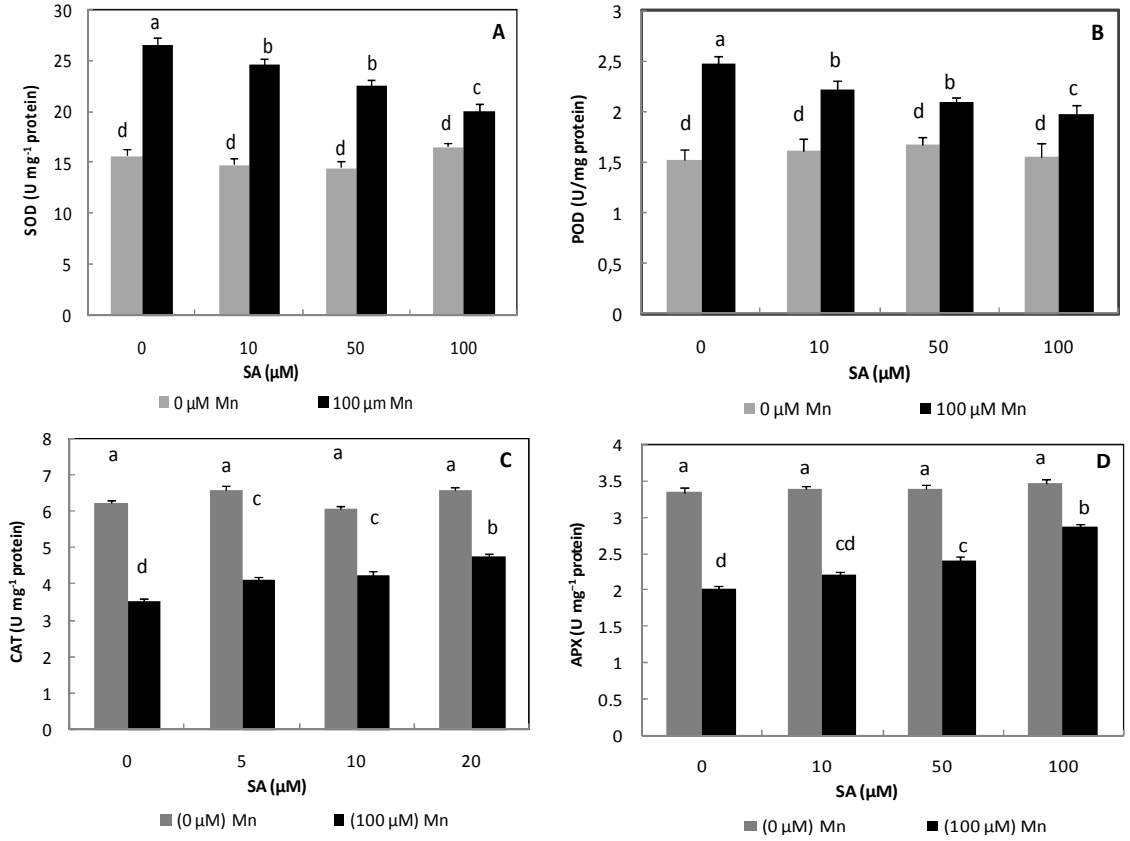

Fig. 1. Effects of SA pretreatment on SOD (A), CAT (B), APX (C) and POD (D) activities in 14-day-old bean plant submitted during 4 days to 100 µM $MnCl_2$. Data are means ± SEM (n=5). Values within rows followed by the same letter(s) are not significantly different according to Tukey's test, (P<0.05)

4. DISCUSSION

In this study, we provide evidence that salicylic acid is able to regulate Mn-induced oxidative stress in bean leaves. Pretreatment with SA before Mn application resulted in significant increase in leaf DM, when compared to Mn-stressed plants grown without SA addition. The ameliorative impact of SA on growth 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 Mn distribution and nutrient uptake [27].

A variety of abiotic stresses, including heavy metals, cause molecular damage to plant cells either directly or indirectly through a burst of active oxygen species (AOS) [28]. These oxygen species (O^-, OH^-, H_2O_2) can convert fatty acids to toxic lipid peroxides, which destroy biological membranes. The present study showed that plant leaves exposed to Mn stress exhibit increased levels of H_2O_2 and MDA as a consequence of the generation of ROS. These findings are consistent with previous reports by Shi and Zhu [29] for cucumber plant, indicating that antioxidant enzymes are not a sufficient defense system.

A drastic increase in H_2O_2 may have as consequence a lower extensibility of plant cell walls, which can rapidly terminate growth [30]. This could explain the decrease of fresh mass observed in bean leaves at 100 µM of Mn. Increased of H_2O_2 content may also inactivate enzymes by oxidizing their thiol groups. However, in Mn-stressed plants pretreated with SA, H_2O_2 level and lipid peroxidation were much lower than in plants treated with Mn only. SA subdued H_2O_2 and MDA formation is supported by several recent reports [29], which indicated the protective effect of SA in lowering H_2O_2 and lipid peroxidation rate under Mn stress conditions.

SA influence on avoiding the toxic effects of Mn may be a consequence of very different primary effects connected with oxidative stress [13] and stabilization of cell membranes [31], leading to the increase of general stress tolerance. SA pretreatment led to a decrease in oxidative injuries as evidenced by decreased H_2O_2 and lipid peroxidation levels. SA may act directly as an antioxidant to scavenge the ROS and/or indirectly modulate redox balance through activation of antioxidant responses. Indeed, it is a direct scavenger of hydroxyl radical and an iron chelating compound, thereby inhibiting the direct impact of hydroxyl radicals as well as their generation *via* the Fenton reaction [32].

The data suggest that endogenous SA plays an important antioxidant role in protecting plants from oxidative stress. In SA-pretreated bean plants, the initially decreased activities of SOD and increased activities of CAT and APX cooperatively controlled the Mn-induced H_2O_2 at high homeostatic levels contrarily to the mode during plant–pathogen interactions [33]. It seems to suggest that SA-reduced H_2O_2 permit bean seedling to respond more effectively to Mn-induced oxidative damage. It is well established that CAT has a high reaction rate but a low affinity for H_2O_2, whereas APX has a high affinity for H_2O_2 and is able to detoxify low concentrations of H_2O_2 [34]. Therefore, it is possible that stimulation of CAT and APX activities by SA decreases the level of H_2O_2 in bean leaves, which may be a possible mechanism in plant defense strategy against Mn-induced oxidative stress.

Oxidative stress is caused by a serious cell imbalance between the production of ROS (H_2O_2), and antioxidative enzymes, which leads to dramatic physiological disorders. The present study has demonstrated that SA alleviates Mn-induced oxidative stress as reflected by reduced H_2O_2, and MDA content. ROS can initiate the peroxidation and destruction of lipid bilayer of cell membrane and consequently affects cell functions. Cell membranes are among the first targets of a number of plant stresses, and the maintenance of membrane integrity and stability is of major importance for stress tolerance [35,36].

The protective role of SA in Mn-Treated plants can be attributed to SA-inhibited lipid peroxidation process, contributing to membrane stability and SA-controlled Mn-induced H_2O_2 at high homeostatic levels by modulating enzymatic (SOD, APX and CAT) activity. SA might influence H_2O_2 signaling pathways in plant defense against Mn, should be further investigated to dissect the complicated network of SA and its involvement in plant defense at a molecular level.

5. CONCLUSION

Based on our results, we propose that the protective role of SA can be related to the decrease in lipid peroxidation, the improvement of scavenging capability of ROS, and the

decrease in Mn uptake in plant. SA may act directly as an antioxidant to scavenge the ROS and/or indirectly modulate redox balance through activation of antioxidant responses.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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