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Exogenous Salicylic Acid Alleviates Zinc-induced Oxidative Damage in Tomato Plants

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Abstract

In the present study, the effects of Zinc (Zn) and salicylic acid (SA) interaction on tomato plants were examined. 25-day-old tomato plants were subjected to different concentrations of $ZnSO_4 . 7H_2O$ (0, 250, 500 µM) and SA (0, 100 µM) for 10 days. Zn was accumulated in roots and shoots of Zn-stressed plants. Treatment with Zn reduced dry weight of roots and shoots and decreased chlorophyll content in leaves. In Zn-treated plants, Malondialdehyde (MDA), H_2O_2 and proline contents significantly increased in leaves. Moreover, changes in the activities of the antioxidant enzymes, catalase, guaiacol peroxidase, ascorbate peroxidase and superoxide dismutase in leaves indicated that Zn caused an oxidative stress in tomato plants. In Zn-stressed plants, application of SA reduced root-to-shoot translocation of Zn and enhanced growth and chlorophyll content. Adding of SA in the nutrient solution, increased the antioxidant enzymes activities and decreased the level of lipid peroxidation, H_2O_2 and proline accumulation in leaves of Zn-treated plants. As a result, SA by sequestration of Zn in roots and increase in the activities of antioxidant enzymes, considerably decreased Zn induced oxidative damage on tomato plants.

Keywords: *Lycopersicon esculentum*, oxidative stress, salicylic acid, zinc toxicity.

INTRODUCTION

Zinc (Zn) is an essential micronutrient needed for plant healthy growth and development. However, at high concentrations Zn can be very toxic to plant cells by interfering with the absorption, transport and homeostasis of essential ions, and the disturbance of metabolic processes [5]. Persistent application of Zn fertilizers as well as its input from industrial pollution resulted in higher Zn content in surface soils, and some crops grown in these soils contain larger amounts of Zn [21]. Symptoms of Zn toxicity comprise the inhibition of growth, modifications of leaf morphology, chlorosis, necrosis and disruption of photosynthesis [27]. Higher Zn concentrations induce oxidative stress by promoting the production of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) [35]. These ROS cause oxidative damage to the vital cellular components like membrane lipids, proteins, enzymes, pigments and nucleic acids, which is damaging to plant growth and development [3]. To scavenge ROS and to prevent oxidative damage, plants possess antioxidative defence system including of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and nonenzymatic antioxidants such as glutathione and ascorbic acid [25]. It has been shown that extra Zn causes oxidative stress in many plant species and changes the activity levels of antioxidative enzymes [6, 7, 11, 20, 26, 30].

Salicylic acid (SA) is considered as a hormone-like endogenous regulator, which plays an important role in the regulation of some physiological processes in plants such as effects on growth and development, ion absorption and transport and membrane permeability [12]. SA acts as a potential non-enzymatic antioxidant and an important signal molecule for modifying plant responses to environmental stressors [15]. It has been shown that exogenous application of SA provides a protection to the plants against the oxidative stresses caused by heavy metals, like cadmium [23], nickel [17] and manganese [33]. SA also changes the activities of antioxidant enzymes and enhances plant tolerance to abiotic stresses [15]. It has been shown that SA has different influences on stress adaptation and damage development of plants that depend on plant species and concentration, method and time of SA application [23].

In recent years, there has been insufficient information about the alleviating Zn toxicity in plants. The objective of the present research was to investigate Zn-induced toxicity in tomato plants and the possible protective role of exogenous SA against the Zn-induced oxidative stress.

MATERIALS AND METHODS

Plant culture and plant treatments

Tomato seeds (*Lycopersicon esculentum* Mill., cv. Urbana VF) were sterilized with sodium hypochlorite solution (1%) for three minutes and then washed extensively with sterile distilled water. Sterilized seeds were germinated on moist filter paper in an incubator at 25ºC for four days. Initially, seedlings with similar sizes were transplanted to 500 ml plastic pots filled with perlite (3 plants per pot) and irrigated with half-strength Hoagland nutrient solution [14] for seven days. The seedlings were then irrigated with full-strength Hoagland solution. Twenty-five-day-old plants were treated with different concentrations of zinc sulphate (0, 250 and 500 µM) without or with SA (0 and 100 µM) in full-strength Hoagland medium for 10 days. The culture and treatment Hoagland solutions were adjusted to pH 6.5 and applied twice a week. The growth of plants was carried out in a culture chamber with 16/8 light/dark photoperiod and irradiance of 190 µmol $m^{-2}s^{-1}$ at the leaf level, day/night temperature of 26º/22ºC and 70% air humidity. After the

treatment with Zn and SA, the samples of roots and shoots were collected and washed with deionized distilled water. For the measure of root and shoot dry weight and zinc content, the samples were dried at 70ºC for 48 h. For the determination of chlorophyll, proline, H_2O_2 and MDA content and enzymatic activities, fresh plant samples were frozen in liquid nitrogen and stored at -70ºC until use.

Determination of zinc content

Zinc content in roots and shoots were analyzed by digestion of dried samples with an acid mixture (HNO_3/$HClO_4$, 4/1, V/V). Zinc was then quantified by atomic absorption spectrophotometry (SpectrAA- 200, Varian, Australia) [7].

Determination of chlorophyll content

Chlorophyll pigment was extracted from 0.2 g leaf fresh weight by 80% acetone. Total chlorophyll contents were determined from absorbance of extract at 646.8 and 663.2 nm according to formula described by Lichtenthaler [19].

Determination of proline

Proline content was estimated by the method of Bates et al. [2]. Leaf sample (0.2 g) was extracted in 10 ml of 3% sulfosalicylic acid. After centrifugation at 10,000g for 10 min, 2 ml of supernatant was reacted with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin and heated for 1 h at 100ºC. The reaction mixture was extracted in 4 ml toluene and the chromophore was sampled from the watery phase. The absorbance was measured at 520 nm against toluene. A standard curve with L-proline was used for the final calculations. Content of proline was expressed as µmol/g FW (fresh weight).

Hydrogen peroxide (H_2O_2) content assay

For estimation H_2O_2 concentration, leaf sample (0.2 g) was extracted with 3 ml 0.1% trichloroacetic acid (TCA) in an ice bath and centrifuged at 12,000g for 15 min [36]. The supernatant (0.5 ml) was added to 0.5 ml of phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the mixture was measured at 390 nm. H_2O_2 content was calculated using the extinction coefficient 0.28 $\mu M^{-1}cm^{-1}$ and the amount expressed as µmol/g FW.

Lipid peroxidation assay

Lipid peroxidation in leaf sample was estimated by measuring the amount of malondialdehyde (MDA), as the product of peroxidation of membrane lipids [13]. Leaf fresh samples (0.2 g) were ground in 3 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000g for 10 min and 1 ml of aliquot was mixed with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was heated at 95ºC for 30 min and then chilled on the ice bath. The consequence of mixture centrifuged at 10,000g for 5 min, and the absorbance of the supernatant was measured at 532 and 600 nm. The value for nonspecific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The content of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as µmol/g FW.

Antioxidant enzymes extraction

For enzyme extracts, 0.5 g tomato leaves were ground in 5 ml of ice-cold potassium phosphate buffer (50 mM, pH 7.5) containing 1 mM Na_2-EDTA, 1% polyvinylpyrrolidone (PVP), with the addition of 5 mM ascorbic acid in the case of ascorbate peroxidase (APX) assay. The homogenate was centrifuged at 15,000g at 4ºC for 30 min. The protein contents in the supernatant were determined according to the method of Bradford [3], with BSA as a standard. The supernatant was collected for the enzyme activity assays.

Antioxidant enzymes assay

The activity of catalase (CAT, EC 1.11.1.6) was determined by following the decomposition of H_2O_2 and decline in absorbance at 240 nm for 1 min [1]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 0.1 ml enzyme extract. The enzyme activity was calculated using the extinction coefficient (39.4 $mM^{-1}cm^{-1}$) and expressed as units (1 µmol of H_2O_2 decomposed per minute) per mg protein.

The activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was measured as a increase in absorption at 470 nm for 3 min due to guaiacol oxidation. The reaction solution consisted of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 0.1 ml enzyme extract [29]. The enzyme activity was calculated using the extinction coefficient (26.6 $mM^{-1}cm^{-1}$) and expressed as units (1 µmol of tetraguaiacol formed per minute) per mg protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined as a decrease in absorbance at 290 nm for 1 min due to ascorbate oxidation [28]. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 ml enzyme extract. The enzyme activity was calculated using the extinction coefficient (2.8 $mM^{-1}cm^{-1}$) and expressed as units (1 µmol of ascorbate oxidized per minute) per mg protein.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring the inhibition of photochemical reduction of nitro blue tetrazoliun (NBT) [9]. The reaction mixture contained 2.4 ml of 50 mM potassium phosphate buffer solution (pH 7.8), 0.2 ml of 195 mM methionine, 0.1 ml of 0.3 mM EDTA, 50 µl enzyme extract, 0.2 ml of 1.125 mM NBT and 0.2 ml of 60 µM riboflavin. The reaction mixture was illuminated for 15 min at light intensity of 5000 lux. The absorbance of solution was measured at 560 nm. One unit of SOD was defined as the amount of enzyme which causes 50% inhibition of the NBT reduction under the assay condition. The enzyme activity was expressed as units per minute per mg protein. All spectrophotometric assays were performed at 25ºC with a UV-vis spectrophotometer (Model UV-190, Shimadzu, Japan).

Statistical data analysis

All data presented here are the mean values of three independent experiments with four replicates. Statistical analysis was performed by analysis of variance (ANOVA) using the SPSS 16.0 software. Mean difference between different treatments was compared by Duncan's test at a 0.05 probability level.

RESULTS

Toxicity symptoms and growth

The presence of both Zn concentration (250 and 500 µM) in the nutrient medium caused visible toxicity symptoms including stunted growth, yellowing roots,

chlorosis and necrosis on the leaves. Addition of 100 µM SA to the nutrient medium particularly in low Zn level (250 µM), markedly decreased the toxic effects of zinc. In Zn-treated plants, with increase of Zn concentration in nutrient medium, Zn content significantly increased in both roots and shoots. In the presence of SA, Zn content increased in roots, but significantly decreased in shoots, as compared to Zn treatments alone (Table 1). The effects of Zn and SA on plant growth, expressed as dry matter of roots and shoots were shown in Table 1. With increase of Zn concentration in culture medium, root and shoot dry weights significantly decreased as compared to the control plants. In the absence of Zn, treatment with SA significantly increased the dry weight of shoots. In Zn-treated plants, application of SA significantly increased both root and shoot dry weight and alleviated the inhibition of plants growth.

Chlorophyll and proline

Tomato plants treated with both Zn level showed a significant decrease in chlorophyll content by 62% and 70% (p<0.001) in low and high Zn levels respectively , as compared to the control plants. The inhibitory effect of zinc on chlorophyll content under SA treatment, was markedly ameliorated (Figure 1a). With increase of Zn level, proline content considerably increased in leaves and concentration of this amino acid in low and high Zn levels was about 2.1 and 3 times (p<0.001) higher than that of control, respectively. Whereas SA significantly decreased proline content in both Zn treatment (Figure 1b).

Table 1. Effects of SA on dry weight and Zn content in roots and shoots of *tomato plants* under zinc stress.

Zn (µM)	SA (µM)	Root Zn content (mg/g dry weight)	Shoot Zn content (mg/g dry weight)	Root dry weight (g/plant)	Shoot dry weight (g/plant)
0	0	0.220 ± 0.011 e	0.306 ± 0.019 d	0.385 ± 0.022 a	1.613 ± 0.076 b
0	100	0.227 ± 0.014 e	0.345 ± 0.018 d	0.418 ± 0.016 a	1.880 ± 0.068 a
250	0	0. 802 ± 0.038 d	0.828 ± 0.060 b	0.207 ± 0.015 c	0.961 ± 0.057 d
250	100	1.093 ± 0.051 c	0.472 ± 0.023 c	0.271 ± 0.010 b	1.356 ± 0.063 c
500	0	1.412 ± 0.071 b	1.461 ± 0.099 a	0.160 ± 0.011 d	0.777 ± 0.047 e
500	100	1.810 ± 0.066 a	0.790 ± 0.054 b	0.210 ± 0.009 c	1.080 ± 0.055 d

Values are the mean ± SE of three experiments with four replicates. The values with the different letters show statistically significant differences between treatments at $p < 0.05$.

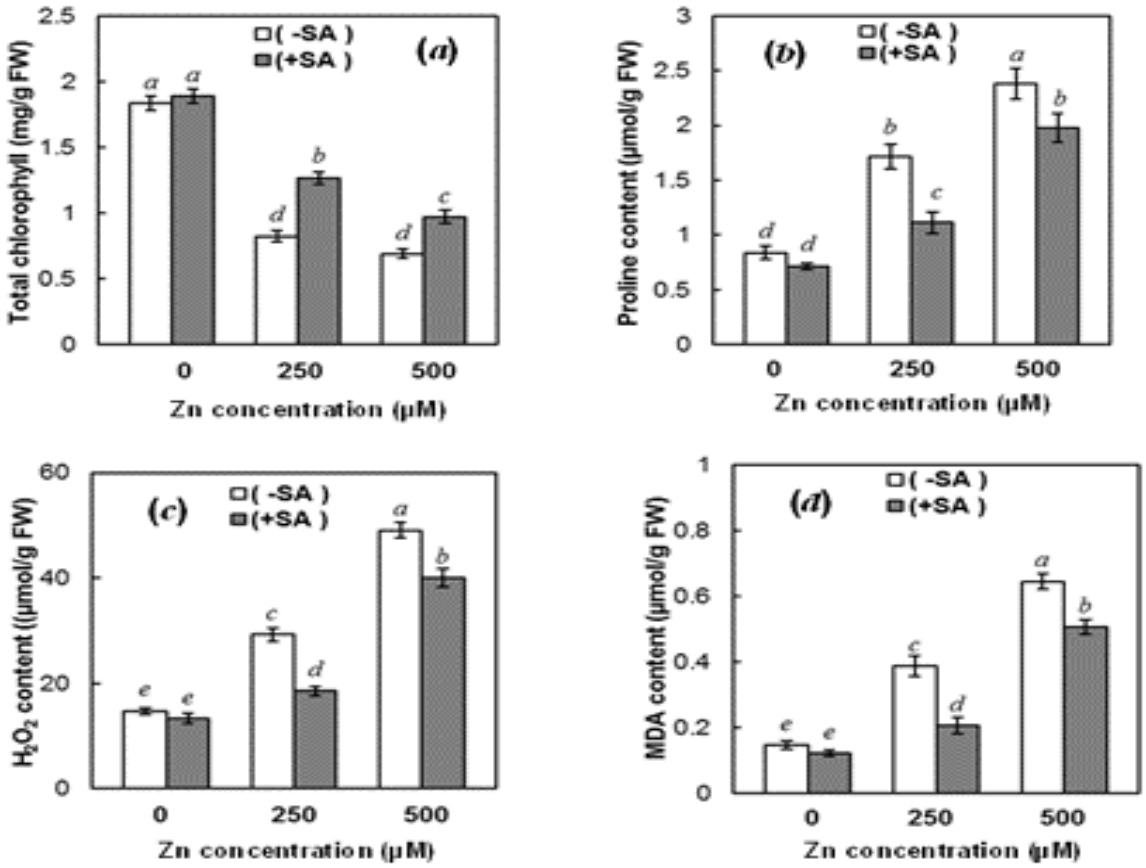

Figure 1. Effects of Zn in presence or absence of SA on total chlorophyll content (**a**), proline content (**b**), H_2O_2 content (**c**) and malondialdehyde (MDA) content (**d**) in leaves. Values are the mean ± SE of three experiments with four replicates. Bars with different letters show significant difference between treatments at $p < 0.05$.

Indicators of oxidative stress

To examine the supposition that extra Zn can cause oxidative stress, H_2O_2 and MDA contents were determined as indicators of oxidative stress. In Zn-treated plants, with increase of Zn concentration, H_2O_2 (Figure 1c) and MDA contents (Fig. 1d) considerably increased in leaves, as compared to the controls. These results show that an obvious oxidative stress happened in the Zn-stressed tomato. Application of SA, significantly reduced H_2O_2 accumulation and MDA production in Zn-stressed plants.

Antioxidant enzymes

Because extra Zn induced oxidative stress in tomato leaves, we next assayed the activity of the antioxidant enzymes. The data in Figure 2a shows that CAT activity was inhibited by 37% and 63% (p<0.001) in leaves of tomato plants treated with 250 and 500 µM Zn respectively, as compared with the controls. When SA were supplemented with zinc ions, CAT activity significantly increased in leaves, as compared with Zn treatments alone. The GPX activity in leaves (Figure 2b) of plants treated with low and high Zn concentrations reduced by 50% and 66% (p<0.001) respectively, as compared to the controls. When plants were treated with Zn either with SA, GPX activity significantly increased in leaves, as compared with Zn treatments alone. In Zn-treated plants APX activity (Figure 2c) in leaves, with increase of Zn concentration were strongly decreased (p<0.001), SA enhanced the enzyme activity in comparison to Zn-treated plants. Contrary to the change of CAT, GPX and APX activity, SOD activity (Figure 2d) in leaves of plants treated with low and high Zn levels markedly enhanced by 42% and 51% respectively over the control level (p<0.001). Application of SA, more increased the SOD activity in plants treated and untreated with zinc sulphate.

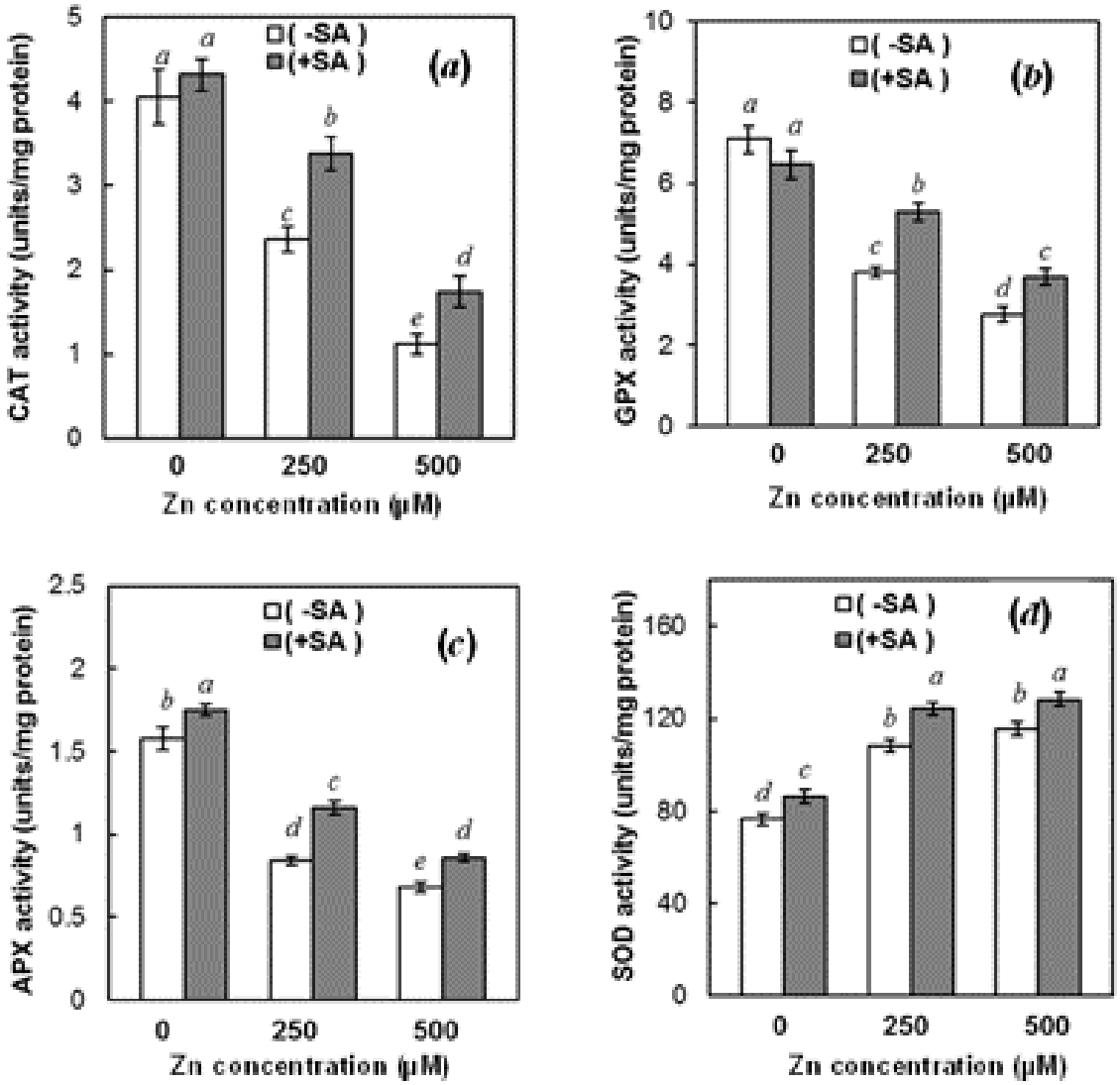

Figure 2. Effects of Zn in presence or absence of SA on catalase (CAT) activity (**a**), guaiacol peroxidase (GPX) activity (**b**), ascorbate peroxidase (APX) activity (**c**) and superoxide dismutase (SOD) activity (**d**) in leaves. Values are the mean ± SE of three experiments with four replicates. Bars with different letters show significant difference between treatments at $p < 0.05$.

DISCUSSION

In plants treated with both Zn concentration (250 and 500 µM), high contents of Zn accumulated in roots and shoots. It is remarkable that SA-treated plants accumulated much less Zn than other plants in shoots, but not in roots. The lower Zn concentrations in the SA-exposed shoots can be caused by decreased Zn absorption and/or by decreased Zn translocation. There was no difference in root Zn content between control and SA-treated plants, thus, SA did not likely affect Zn absorption by roots. We suggested that SA reduced the root-to-shoot translocation of Zn, therefore resulting in low Zn accumulation in shoots. Similar result has been discovered in Ni-stressed canola [17]. Contrary to our result, exogenous SA reduced the Ni content in roots and enhanced in shoots of *Matricaria chamomilla* plants under Ni stress [18]. However, SA reduced Cd absorption in Cd-stressed maize plants [12]. Symptoms of Zn toxicity such as chlorosis and necrotic spots were markedly obvious in tomato plants treated with both Zn concentration alone. Many authors reported reduced chlorophyll contents in the leaves of Zn-treated plants [6, 7, 11, 16, 31, 34]. Such chlorosis could result from both Fe and Mg deficit and the inhibition of chlorophyll synthesis [31]. We observed chlorosis of leaves, reduction in chlorophyll content and decreased dry biomass of shoot and root tissues. Inhibitory effect of Zn on plant growth has been reported by several authors [7, 16, 21, 30, 31]. Inhibition of growth in tomato plants might result from Zn-caused change of essential metabolic processes, e.g. photosynthesis and transport of photoassimilates from leaves [21]. Application of SA, could significantly improve the growth and chlorophyll content of tomato plants exposed to Zn stress. Similar result has been found in Ni-stressed canola [17]. Treatment with SA alone enhanced the dry weight of shoots in tomato plants. Similarly, enhancement of growth by SA has also been reported in *Matricaria chamomilla* plants [18]. We observed that SA ameliorates Zn toxicity on growth and chlorophyll content. In agreement with this, SA was also reported to stimulate the photosynthetic machinery and enhance the chlorophyll content and growth of sunflower plants under Cu stress [8]. SA could also be concerned with the regulation of cell enlargement and division in synergy during plant development [15]. Zinc, similarly to other heavy metals, causes the production of ROS in plants [35]. In the present research, the tomato plants reacted against Zn treatments with increase of H_2O_2 content in leaves. The enhancement of H_2O_2 content in response to Zn stress has been found in radish seedlings [30], roots and leaves of wheat plants [20].

It has been suggested that H_2O_2 plays an important role in the inhibition of growth of heavy metal-stressed plants [27]. As substrate for peroxidases it take parts in hardening of cell walls, which may lead to the limitation of cell elongation. Hydrogen peroxide has also been found to negatively affect proliferation of cells [32]. The Zn-induced oxidative stress in tomato plants was obvious from the enhanced lipid peroxidation in leaves.

In comparison to control plants, Zn treatments in the absence of SA resulted in enhanced MDA content. These results are in agreement with those reported by other authors [6, 7, 11, 20, 26, 30]. High level of H_2O_2, which stimulates Haber-Weiss reaction, resulting in $^{\bullet}OH$ formation followed by lipid peroxidation [25]. Our results indicated that SA reduced H_2O_2 and MDA accumulation caused by Zn toxicity. The decrease in MDA and H_2O_2 content by SA treatment, has been reported in rice plants under Cd stress [10] and canola leaves treated with Ni [17]. The enhancement of CAT, GPX, and APX activities by SA, decreased much production of ROS such as $O_2^{\bullet-}$, H_2O_2 and so on [12]. Tomato plants reacted to Zn exposure with accumulation of proline, which is in agreement with other reports on the effect of Zn on plants [20, 24, 26, 30]. It has been shown that Zn accumulation may result in disturbance of plant water balance [31]. Therefore, proline accumulation found in our experiment may be related to the mechanisms of osmoregulation, as well as, the important role of proline in response of plants to heavy metal toxicity may be concerned with its antioxidative properties [22]. In our research, SA appears to be able of moderating damage associated with Zn stress by decreasing oxidative stress and inducing proline degradation. The observed reduction in the level of proline in SA-treated plants showed partial alleviation from Zn stress. The decrease in proline concentration by SA pretreatment has been reported in barley plants under Cd stress [23]. It has been proposed that non redox metals, comprising Zn, may induce oxidative stress indirectly, by reducing the efficiency of antioxidative system [30]. Treatment of the tomato plants with both Zn concentration significantly reduced the activities of CAT, GPX and APX. These enzymes are responsible for the scavenging of H_2O_2 in plants [25]. The decrease in their activities induced oxidative damage in leaves of Zn-stressed plants. Data concerning the effect of Zn treatment on CAT, GPX and APX activities in plants are contradictory, since both enzymes activation [20, 34] and inhibition [6, 30] have been reported. These enzymes (CAT, GPX and APX) contain Fe in their structure. Since high concentrations of Zn have been shown to reduce Fe content in plant tissues [31], it can be supposed that decrease in their activities in leaves of tomato plants exposed to extra Zn may result from deficit of Fe for biosynthesis of these enzyme molecules. The reduction in antioxidative enzyme activities could also be due to disturbance of enzyme molecules by toxic ROS [25]. The reduced activities of these three enzymes show that they may not play essential roles in detoxifying ROS under Zn stress. Contrary to CAT, GPX and APX, SOD activity in the tomato leaves enhanced in response to Zn treatments, which is in agreement with the findings for tomato [7] and wheat [20] plants. In contrast, inhibition of this enzyme activity has also been reported in radish seedlings under Zn stress [30]. SOD catalyzes disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2 and is considered to be a key enzyme in protecting cells against oxidative stress [25]. Increased activity of SOD in present study indicates its important role in removing $O_2^{\bullet-}$ generated due to Zn stress. In present study, when SA was applied simultaneously with Zn ions, this substance enhanced CAT, GPX, APX and SOD activities and reduced MDA and H_2O_2 content. The physiological role of SA is usually considered to be as a signal molecule, changing the antioxidative system by inhibiting CAT and stimulating peroxidases [15]. In our research, the presence of SA in Zn-stressed plants enhanced the activity of catalase (CAT) and peroxidases (GPX, APX). Similar studies relating to enhancing of these enzymes activity (CAT, GPX and APX) by treatment with SA has been reported in leaves of *canola plants* under Ni stress [17] and Cd-exposed rice plants [10]. The adding of SA increased SOD activity under Zn stress and therefore enhanced the ability of $O_2^{\bullet-}$ scavenging. Similarly, stimulation of SOD activity by SA has been

observed in Mn-stressed cucumber plants [33]. This suggested that stimulation of antioxidants might be achieved by SA-induced protein synthesis [12]. It has been shown that SA acts as a signaling molecule in regulating H_2O_2 levels associated with H_2O_2-metabolizing enzymes [15]. In the present study, treatment with SA stimulated the activities of CAT, GPX and APX and reduced H_2O_2 and MDA levels in leaves. In agreement with our findings, exogenous SA decreased H_2O_2 and MDA contents and ameliorated the membrane damage induced by Ni in canola plants [17]. SA with chelating action on metals, may reduce MDA content in plants under heavy metal stress. Therefore, SA participates in the stabilization of cell membranes [15] and with enhancement of antioxidant enzymes activities in leaves, leading to alleviation of the oxidative damage as indicated by the lowered H_2O_2 and MDA levels.

CONCLUSION

In accordance with the results of this study, reduction of zinc toxicity in SA-treated plants may be described by low Zn accumulation in shoots of these plants due to inhibition of Zn translocation from roots to shoots or sequestration of Zn in roots and also with activation of antioxidant enzymes reducing Zn-induced oxidative stress.

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