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Effects of exogenous salicylic acid and sodium nitroprusside on α-tocopherol and phytochelatin biosynthesis in zinc-stressed safflower plants

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Abstract: The interactive effect of exogenous application of salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on zinc (Zn) toxicity was assessed in Zn-stressed safflower (*Carthamus tinctorius* L.) seedlings. Exposure to 500 µM $ZnSO_4.7H_2O$ for 10 days caused an increment in the levels of malondialdehyde (MDA), H_2O_2, α-tocopherol, phytochelatins (PCs), and proline. Application of SA or SNP, and especially their combination, resulted in remarkable decrease in MDA, H_2O_2, and proline content while chlorophyll content and the activity of catalase, ascorbate peroxidase, and guaiacol peroxidase exhibited a significant induction in comparison with plants subjected to Zn treatment alone. In Zn-treated plants, addition of SA and SA + SNP caused a progressive increase in α-tocopherol levels in comparison with plants treated with Zn alone. In plants subjected to Zn excess, no significant association was found between PC levels and the supplementation of SA, while application of SNP and SA + SNP caused a significant increase in PC contents. These data imply that SA and SNP, and especially in combination, play a significant role in the amelioration of deleterious effects of Zn toxicity, probably due to stimulation of antioxidative defense mechanisms and PC biosynthesis.

Key words: Nitric oxide, oxidative stress, phytochelatin, safflower, salicylic acid, zinc

1. Introduction

Zinc, the second most abundant transition metal after iron, is an essential element for normal growth and development at low concentrations. Zinc performs a substantial role as a cofactor for several enzymes and contributes to protein synthesis, as well as carbohydrate, nucleic acid, and lipid metabolism. However, elevated levels of Zn can cause the creation of excess reactive oxygen species (ROS), which influences plant growth and development (Kazemi et al., 2010; Li et al., 2013; Wang et al., 2013). Inductions in the levels of oxidative stress markers like malondialdehyde (MDA), H_2O_2, and the activity of lipoxygenase (LOX), an enzyme responsible for lipid peroxidation, have been reported under heavy metal stress (Gill and Tuteja, 2010). Plants have evolved several mechanisms to combat ROS-caused oxidative damage. These include antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), and low-molecular-mass scavengers such as glutathione (GSH), α-tocopherol, and proline (Gill and Tuteja, 2010; Subba et al., 2014).

Proline, a proteinogenic five-carbon α-amino acid, is among the foremost scavengers of ROS identified as indicators of several kind of environmental stress, like salinity and heavy metals (Li et al., 2013). It has also been reported that proline can act as a metal chelator (Liang et al., 2013).

Metalloenzyme SOD operates as a first line of defense against ROS-induced injury, catalyzing the dismutation of highly reactive $O_2^{\bullet-}$ to O_2 and H_2O_2. The resulting H_2O_2 is quickly averted by APX in the ascorbate-glutathione cycle or through other enzymes like GPX and CAT into cytoplasm and other cellular compartments (Gill and Tuteja, 2010). CAT, a heme-containing enzyme, is responsible for the dismutation of H_2O_2 into H_2O and O_2. GPX is able to decompose indole-3-acetic acid and is responsible for lignin production. This enzyme is involved in defense against various stresses by consuming H_2O_2 (Gill and Tuteja, 2010).

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Alpha-tocopherol, a classical lipophilic antioxidant existing in green parts of plants, belongs to tocopherols that are commonly known as vitamin E. These potent antioxidants are among the most powerful ROS scavengers that are able to quench the singlet oxygen (Gill and Tuteja, 2010).

Phytochelatins (PCs) are low-molecular-weight metal-binding, cysteine-rich polypeptides with the general structure of (γ-Glu-Cys)nGly (n = 2–11), which generate complexes with metals and sequester them into plant cell vacuoles, resulting in metal detoxification in cells (Batista et al., 2014). Phytochelatin synthase enzyme is responsible for PC biosynthesis where it catalyzes PC synthesis from GSH in the presence of certain metals. There are several studies regarding the vital role of PCs in heavy metal detoxification (Mishra et al., 2006; Tennstedt et al., 2009).

During the last decade, the application of signaling compounds has increased, improving the health of plants against metal toxicity. There are several reports associated with the important role of signaling molecules in improving heavy metal tolerance by the modification of antioxidant systems (Kazemi et al., 2010; Mostofa and Fujita, 2013; Wang et al., 2013). Salicylic acid (SA) is a natural signaling molecule that performs an important role in plant resistance to a number of environmental stresses by the modulation of several physiological procedures. The noticeable role of SA application on alleviation of destructive effects of heavy metals stress is well established (Dong et al., 2015).

Several studies have shown the important role of nitric oxide (NO), a gaseous signaling molecule, in modulating many vital physiological processes in plants. The application of SNP, a donor of NO, has been stated to provide protection against the destructive effects of heavy metal stress on plants (Kazemi et al., 2010).

Among plants of the family Asteraceae, safflower (*Carthamus tinctorius* L.) has been considered as an important crop with broad geographical distribution, which can endure environmental stress, such as heavy metal stress (Namdjoyan et al., 2012; Srivastava and Bhagyawant, 2014; Namdjoyan et al., 2017). Although there are several studies pertaining to safflower's tolerance to heavy metal stress and also to the role of SA and NO as signaling molecules of antioxidant defense systems in heavy metal-stressed plants, to the best of our knowledge, there are no studies considering the interactive effects of SA and NO on antioxidative responses of safflower seedlings under Zn toxicity. Additionally, to our knowledge, the interactive effects of SA and NO on PC synthesis have not yet been assessed. Hence, the aims of this study were to perform a comparative analysis of the possible effects of the exogenous SA and SNP (as NO donor) on (1) the alleviation of Zn-induced toxicity and (2) the antioxidative responses with reference to proline and α-tocopherol content and antioxidant enzyme activity along with PC synthesis of safflower seedlings under Zn toxicity.

2. Materials and methods

2.1. Plant material and growth conditions

Safflower (cv. Arak2811) seeds were obtained from the Seed & Plant Certification & Registration Institute, Karaj, Iran. After sterilization for 8 min with 0.1% mercuric chloride (w/v), seeds were germinated between two layers of moistened filter paper for 48 h at 25 °C. After germination, seedlings were transplanted into plastic pots (volume: 500 mL), 3 plants per pot, filled with perlite and watered with Arnon and Hoagland's full-strength nutrient solution (Arnon and Hoagland, 1940). After 3 weeks, seedlings were exposed to Zn treatments (0 and 500 μM $ZnSO_4.7H_2O$) without or with SA or SNP (0 and 100 μM) as follows: (1) control (0 μM SA + 0 μM SNP + 0 μM $ZnSO_4.7H_2O$), (2) SA (100 μM SA + 0 μM SNP + 0 μM $ZnSO_4.7H_2O$), (3) SNP (0 μM SA + 100 μM SNP + 0 μM $ZnSO_4.7H_2O$), (4) SA + SNP (100 μM SA + 100 μM SNP + 0 μM $ZnSO_4.7H_2O$), (5) Zn (0 μM SA + 0 μM SNP + 500 μM $ZnSO_4.7H_2O$), (6) SA + Zn (100 μM SA + 0 μM SNP + 500 μM $ZnSO_4.7H_2O$), (7) SNP + Zn (0 μM SA + 100 μM SNP + 500 μM $ZnSO_4.7H_2O$), (8) SA + SNP + Zn (100 μM SA + 100 μM SNP + 500 μM $ZnSO_4.7H_2O$). Preliminary experiments with different concentrations of $ZnSO_4.7H_2O$ (0, 100, 250, 500 μM), SA (0, 50, 100, 200 μM), and SNP (0, 50, 100, 200 μM) were carried out independently to determine the appropriate test concentrations (data not presented). Hoagland solution (pH 6.5) was renewed at least every 2 days. Plants were kept at 26/22 °C day/night temperature under a 16/8 light/dark photoperiod, photon flux density about 200 μmol m^{-2} s^{-1}, and relative humidity ranging between 65% and 70%. After 10 days, plant samples were used for the study of different parameters.

2.2. Chlorophyll contents determination

Chlorophyll measurement was performed by homogenization of fresh leaf tissues (0.2 g) in 80% (v/v) acetone. Homogenates were then filtered and adjusted to a final volume of 20 mL. Total chlorophyll contents were determined spectrophotometrically at 645 and 663 nm using the procedure described by Lichtenthaler (1987).

2.3. Analysis of lipid peroxidation

Lipid peroxidation was measured according to Heath and Packer (1968) by evaluation of the content of MDA in tissues. After centrifugation of fresh leaf tissues (0.5 g) at 10,000 × *g* for 5 min in 5 mL of 0.1% trichloroacetic acid (TCA), to every 1 mL of aliquot, 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added. The mixture was incubated at 95 °C for 30 min and then cooled quickly on ice, and finally the absorbance of centrifuged

supernatant was read at 532 and 600 nm (E= 155 mM^{-1} cm^{-1}).

2.4. Hydrogen peroxide (H_2O_2) determination

Hydrogen peroxide content was measured spectrophotometrically in accordance with the method of Velikova et al. (2000). The extraction was done using 0.2 g of fresh samples in 3 mL of 0.1% (w/v) TCA in a bath. After centrifugation for 15 min at 12,000 × g, 0.5 mL of supernatant was added to 0.5 mL of phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The content of H_2O_2 was determined by the estimation of absorbance at 390 nm (E= 0.28 μM^{-1} cm^{-1}) and stated as μmol g^{-1} FW (fresh weight).

2.5. Lipoxygenase (LOX) activity assays

Lipoxygenase (EC 1.13.11.12) activity was evaluated by the method of Doderer et al. (1992). About 0.2 g of fresh leaf tissues was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 μM EDTA using a prechilled pestle and mortar. After centrifugation at 15,000 × g for 15 min at 4 °C, the resulting supernatant was used as enzyme extract. The substrate solution was prepared by mixing 35 μL of linoleic acid, 5 mL of distilled water, and 50 μL of Tween-20. After adjusting the pH to 6.5, 0.1 M phosphate buffer (pH 6.5) was supplemented to make a total volume of 100 mL. After adding 50 μL of enzyme extract to 2.95 mL of substrate, LOX activity was measured. The solution absorbance was recorded at 234 nm (E = 25 mM^{-1} cm^{-1}).

2.6. Antioxidant enzyme activity assays

Fresh leaf samples (0.5 g) were homogenized with a mortar and pestle in 5 mL of ice-cold potassium phosphate buffer (50 mM, pH 7.5) containing 1 mM ethylenediamine tetra acetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). After homogenization in cold phosphate buffer, the homogenate was centrifuged at 15,000 × g at 4 °C for 15 min to remove plant debris. The supernatant was used for the following assays of antioxidant enzyme activities.

The protein contents were estimated in accordance with Bradford (1976), using BSA as a standard. For the estimation of SOD (EC 1.15.1.1) activity, 1.5 mL of reaction mixture was prepared by mixing 100 mM potassium phosphate buffer (pH 7.5), 13 mM methionine, 2.25 mM nitroblue tetrazolium (NBT), 0.1 mM EDTA, 60 μM riboflavin, and a suitable aliquot of enzyme extract. Amount of enzyme required to cause 50% reduction in color at 560 nm was expressed as one unit of SOD activity (Beauchamp and Fridovich, 1971).

CAT (EC 1.11.1.6) activity was measured using a mixture containing 25 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 10 mM H_2O_2, and the enzyme. A decrease in the absorbance of H_2O_2 at 240 nm within 1 min (E = 39.4 mM^{-1} cm^{-1}) was recorded (Aebi, 1984).

APX (EC 1.11.1.11) activity was measured by the method of Nakano and Asada (1987). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, and the enzyme aliquot. The oxidation of ascorbate was estimated at 290 nm (E = 2.8 mM^{-1} cm^{-1}).

For the estimation of the activity of GPX (EC 1.11.1.7), a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2, and enzyme was prepared (Polle et al., 1994). One unit of enzyme is defined as the volume of enzyme necessary for oxidation of 1 μmol of substrate per minute at 470 nm (E = 26.6 mM^{-1} cm^{-1}).

2.7. Proline determination

After homogenization of 0.2 g of leaf samples in 10 mL of 3% aqueous sulfosalicylic acid, homogenates were centrifuged at 10,000 × g for 10 min. Supernatant (2 mL) was then added to a tube containing 2 mL of glacial acetic acid and 2 mL of acid ninhydrin and mixed well for 1 h at 100 °C. After addition of 4 mL of toluene, the generated color was determined at 520 nm. The content of proline was calculated using a standard curve prepared by L-proline (Bates et al., 1973).

2.8. Estimation of α-tocopherol content

The α-tocopherol content was measured by the method of Backer et al. (1980). For this, 0.5 g of fresh leaf tissues was homogenized in 10 mL of a mixture of petroleum ether and ethanol (2:1.6, v/v). After centrifugation at 10,000 × g for 20 min, 0.2 mL of 2% 2,2-dipyridyl in ethanol was added to 1 mL of extract and mixed well. After incubation for 5 min in the dark, the resultant product was mixed with 4 mL of distilled water. The color was measured at 520 nm and the α-tocopherol content was calculated using a standard graph prepared with known volumes of α-tocopherol.

All spectrophotometric analyses were performed using a Shimadzu model UV-1601 PC UV-Vis spectrophotometer at 25 °C.

2.9. Extraction and assay of PCs

The determination of PC content was performed by the method of Molina et al. (2008) with a slight modification. In brief, fresh leaf samples (0.5 g) were ground in 3 mL of 3% (w/v) sulfosalicylic acid for the extraction of nonprotein thiols (NP-SH). The supernatant was centrifuged at 10,000 × g for 15 min at 4 °C and then was immediately mixed with Ellman's reaction mixture containing 5 mM EDTA and 0.6 mM 5,5 *0*-dithiobis (2-nitrobenzoic acid) in 120 mM phosphate buffer (pH 7.5). The absorbance was read after 5 min at 412 nm to determine the level of NP-SH. GSH contents were measured by the recycling method of Anderson (1985). The difference between total nonprotein SH and GSH was considered in theoretical determination of PCs.

2.10. Statistical analysis

Values reported are the means with standard error (SE) of five independent replicates. SPSS 17.0 was used. ANOVA was done to assess the mean difference comparison between different treatments, followed by Duncan's multiple range test at a 0.05 probability level.

3. Results

In this study, plants subjected to Zn treatment exhibited a significant decrease in total chlorophyll content (79%) in comparison with the control. However, the addition of SA, SNP, and their combination caused a dramatic increase in chlorophyll content compared to Zn treatment alone (Figure 1). A 73% increase in chlorophyll content was noted in Zn-stressed plants treated with SA + SNP in comparison with plants subjected to Zn alone (Figure 1).

A 54% induction in the MDA content was recorded in plants treated with Zn in comparison with the control (Figure 2a). However, under supplementation of SA, SNP, and especially the combination of SA and SNP, the content of MDA remarkably decreased in comparison to Zn treatments alone (Figure 2a).

The content of H_2O_2 increased progressively in the leaves of Zn-treated safflower plants with respect to the controls. Adding SA, SNP, and in particular their combination revealed a sharp depletion in H_2O_2 content of Zn-stressed plants (Figure 2b).

In comparison with the control, the activity of LOX in leaves of plants exposed to Zn increased significantly. Compared to the control, Zn treatment caused a 52% increase in LOX activity. Adding SA, SNP, or the combination of these molecules caused a sharp decrease in LOX activity in the leaves as compared to plants subjected to Zn-only treatment (Figure 2c).

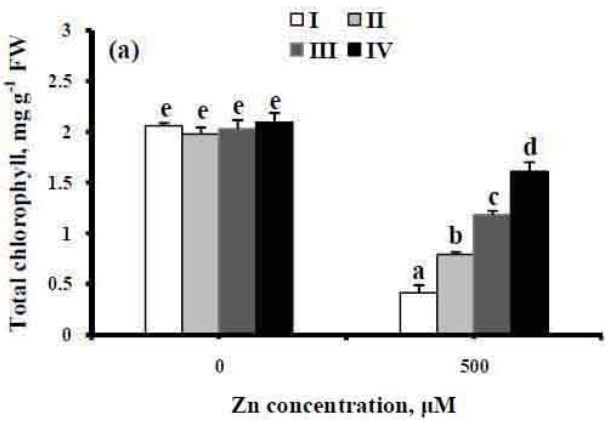

Figure 1. Effects of Zn in combination with SA and SNP on total chlorophyll content. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Bars represent the mean ± SE of three experiments with five replicates. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

In comparison with the control, Zn treatment alone caused a 3.6-fold increase in SOD activity. However, upon supplementation with SA, SNP, and their combination, the activity of SOD decreased significantly with respect to Zn treatment alone (Figure 3a).

The activities of CAT, APX, and GPX exhibited an analogous trend in response to Zn treatment. Treatment with Zn alone caused a significant decline in the activities

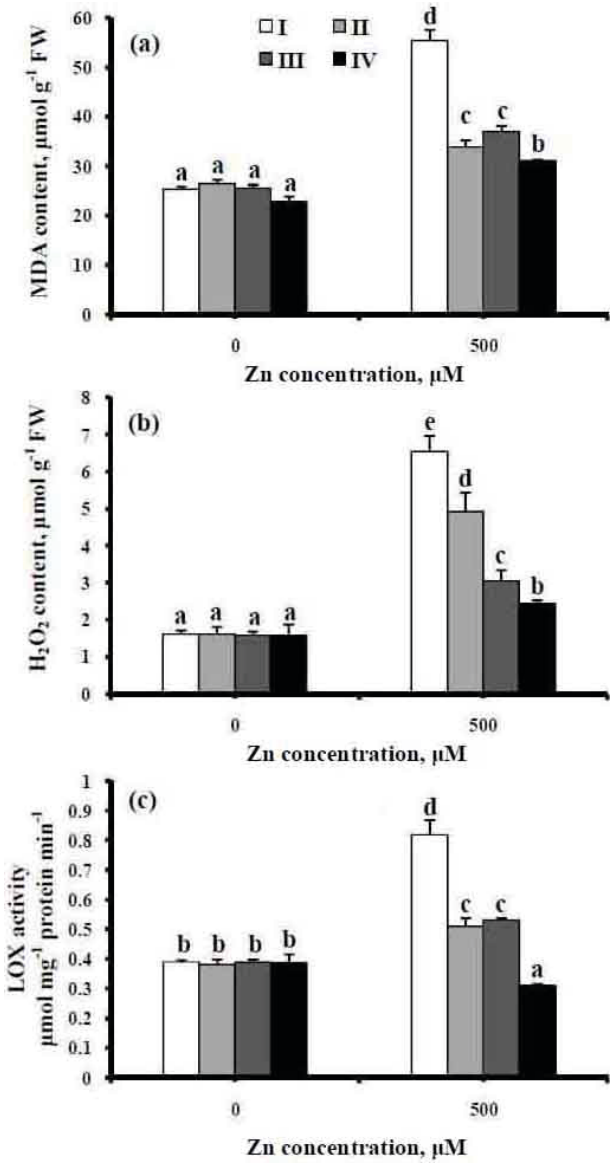

Figure 2. Effects of Zn in combination with SA and SNP on oxidative stress markers, MDA content (a), H_2O_2 content (b), and LOX activity (c) in leaves. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Bars represent the mean ± SE of three experiments with five replicates. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

of CAT, APX, and GPX in comparison with the control. In the case of CAT and APX, the enzyme activity reduced significantly when compared to all of the plant groups grown in the absence of Zn (Figures 3b–3d). However, adding SA, SNP, and SA + SNP caused a significant induction in the activities of CAT and APX in comparison with the plants treated only with Zn (Figures 3b and 3c). Interestingly, upon application of SA, SNP, and SA + SNP, the activity of GPX exhibited a sharp increment with respect to Zn treatment alone. As compared with all of the plant groups grown in the absence of Zn, GPX activity increased remarkably in SA-, SNP-, and SA + SNP-supplemented Zn-treated plants (Figure 3d).

As shown in Figure 4a, the proline content in the leaves of Zn-treated plants was enhanced by 47% ($P \leq 0.05$) with respect to the control. However, the supplementation of SA, SNP, and their combination caused a significant depletion in the proline content of Zn-stressed plants in comparison with Zn treatment alone.

When compared to the control, the content of α-tocopherol increased significantly under Zn treatment alone (Figure 4b). A 5.1-fold increase in α-tocopherol content was noted in the leaves of plants exposed to Zn alone. Further increase of α-tocopherol content was recorded when SA and its combination with SNP were added to the plants exposed to Zn excess (Figure 4b). A 28% induction in the α-tocopherol content was noted in SA + Zn-treated plants in comparison with plants treated only with Zn (Figure 4b).

As shown in Figure 5, the PC content in the leaves of Zn-treated plants increased remarkably as compared to the control. Further increment of the PC content was noted when SNP and the SA + SNP combination were added to plants subjected to Zn excess (Figure 5). Compared to the plants exposed to Zn treatment alone, a 38% increase in the content of PC was recorded in SNP + Zn-treated plants (Figure 5).

4. Discussion

In this study, exposure to Zn stress caused a significant decrease in the chlorophyll contents of Zn-treated plants. Our results are in agreement with a number of reports on

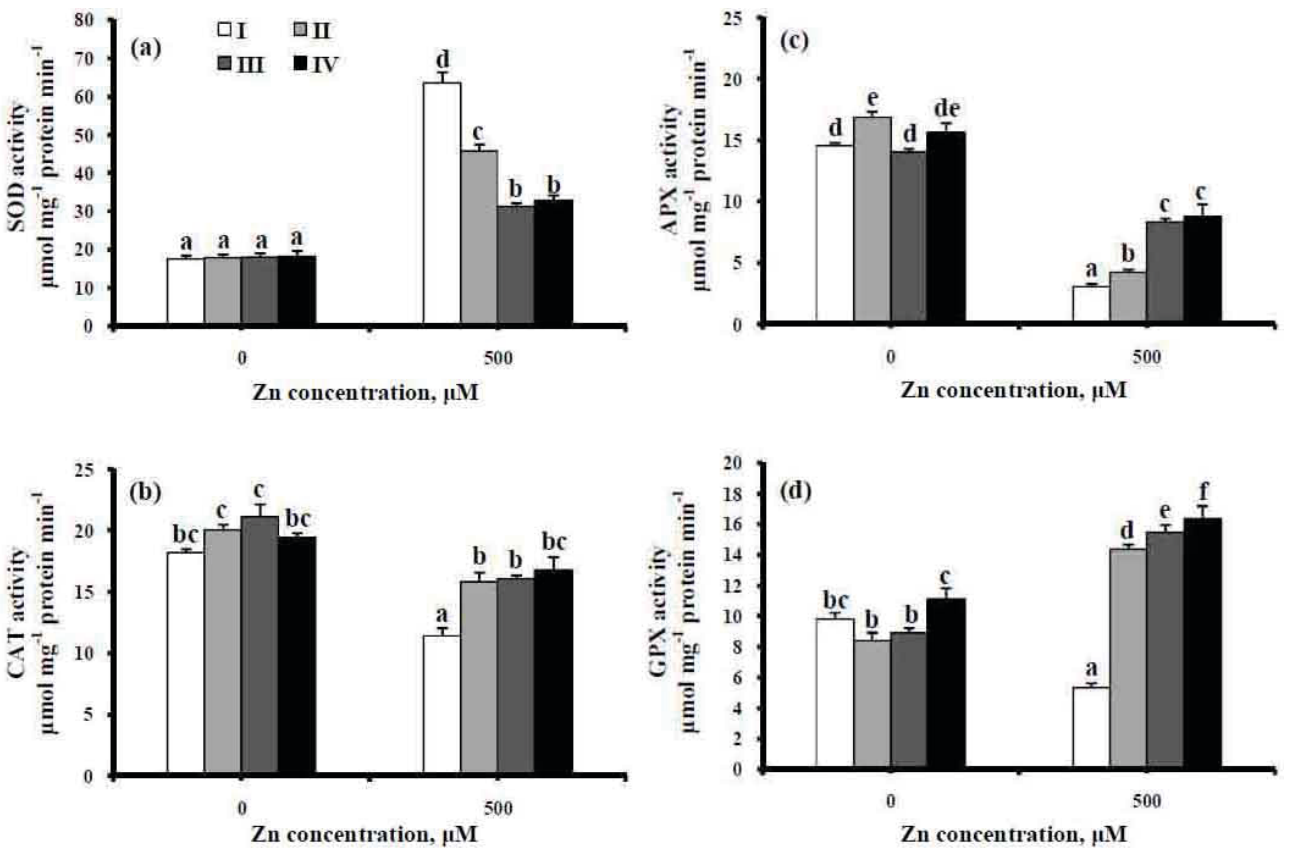

Figure 3. Effects of Zn in combination with SA and SNP on SOD activity (a), CAT activity (b), APX activity (c), and GPX activity (d) in leaves. (I) Without SA and SNP, (II) 100 μM SNP, (III) 100 μM SA, and (IV) 100 μM SA + 100 μM SNP. Bars represent the mean ± SE of three experiments with five replicates. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

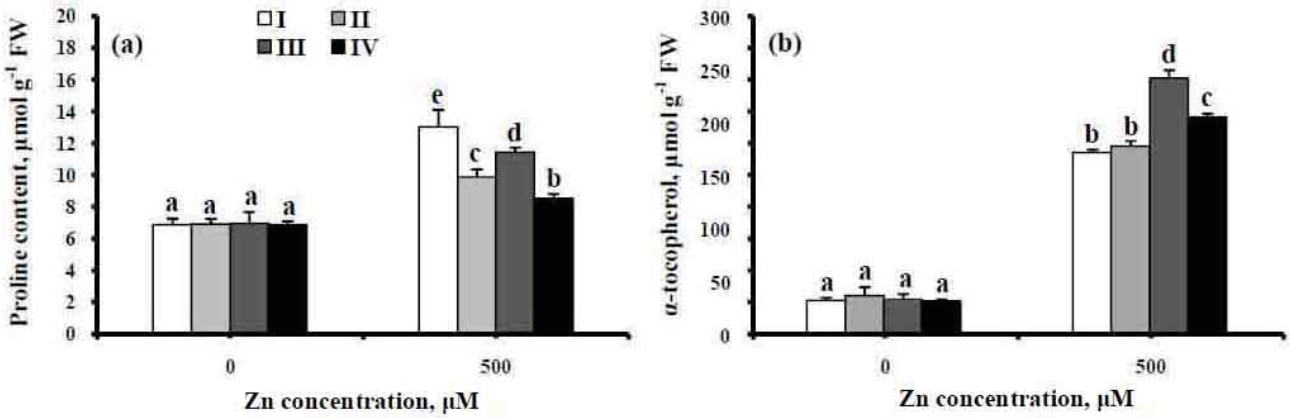

Figure 4. Effects of Zn in combination with SA and SNP on proline (a) and α-tocopherol (b) content in leaves. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Bars represent the mean ± SE of three experiments with five replicates. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

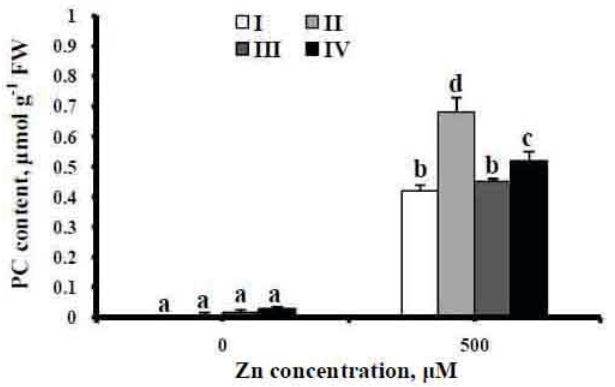

Figure 5. Effects of Zn in combination with SA and SNP on PC content in leaves. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Bars represent the mean ± SE of three experiments with five replicates. Different letters indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

plants exposed to heavy metal stress (Kazemi et al., 2010; Wang et al., 2013). The decrease in chlorophyll content under heavy metal stress could be associated with the inhibition of enzymes involved in chlorophyll biosynthesis and/or the inhibition of uptake and transportation of some metal elements like Mn and Fe (Kazemi et al., 2010; Yadav 2010; Anwaar et al., 2015). We also suggest that decreased chlorophyll content under Zn stress may be associated with the efficient generation of ROS and their adverse effects on the photosynthetic transport chain. In agreement with our results, SA and SNP have been reported to improve chlorophyll content by reducing the level of ROS (Sing et al., 2008; Lee and Park, 2010). Furthermore, it has been reported that SA and NO as signaling molecules are able to mediate Fe deficiency and increase chlorophyll content (Wang et al., 2013).

In our experiment, MDA content and LOX activity of Zn-stressed plants increased markedly. Additionally, a significant increase in the level of H_2O_2 in plants treated with Zn alone was noted. These changes reveal the extent of the oxidative stress induced by Zn toxicity in Zn-stressed safflower plants. Similar to our results, increased MDA and H_2O_2 content and also enhanced LOX activity were shown in some plant species under heavy metal stress (Saxena and Shekhawat, 2013; Wang et al., 2013). The increment in the level of MDA is associated with the degree of lipid peroxidation. The greater lipolytic activity and enhanced oxidation of membrane-bound fatty acids under stress conditions like heavy metal stress are associated with the elevated stimulation of LOX activity (Hasanuzzaman et al., 2017). However, upon supplementation of SNP, SA, or the combination of these molecules, the levels of MDA and H_2O_2 and also the activity of LOX sharply decreased in safflower plants exposed to Zn excess. SNP + SA was found to be more efficient in decreasing the level and activity of these oxidative stress markers, implying a more drastic membrane-preserving role for SNP + SA treatment. Previously, Wang et al. (2013) suggested that reduced aggregation of ROS might be indirectly attributed to the NO-mediated enhanced activity of antioxidant enzymes. Moreover, it has been shown that NO can scavenge lipid peroxyl radicals and inhibit lipid peroxidation, and therefore it is capable of protecting membrane unity. It has been reported that SA can alleviate heavy metal-induced injury through the chelation of metal and also by increasing the antioxidant enzymes activities (Mostafa and Fujita, 2013; Szalai et al., 2013).

In this work, there was a significant increase in SOD activity of Zn-treated plants. Since SOD as a key enzyme

is responsible for protecting cells against oxidative stress, through dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2, it seems that the Zn-induced accumulation of H_2O_2 in Zn-treated safflower plants may be, at least in part, due to Zn-elevated SOD activity. Enhanced ROS production and increased expression of SOD encoding genes may also be responsible for the elevated activity of SOD. However, SNP-, SA-, and SNP + SA-treated plants revealed a remarkable decrease in SOD activity, showing the important role of these signaling molecules in ROS quenching. Elimination of SOD activation can be explained by NO itself being a direct scavenger of superoxide radicals (Bavita et al., 2012).

In the present investigation, it is worth noting that the activities of APX, CAT, and GPX sharply decreased in Zn-stressed plants. Our results are in agreement with several other studies that showed depletion in APX and CAT activity after heavy metal treatments (Kazemi et al., 2010; Wang et al., 2013). Enzymes such as APX, CAT, and GPX incorporate Fe in their structure. Since Zn stress has been shown to reduce Fe uptake and induction of iron deficiency (Pahlsson, 1989; Yadav, 2010), we suggest that Fe deficiency may be associated with the depletion of the activities of APX, CAT, and GPX in plants treated with Zn alone. A decrease in CAT activity in Zn-stressed safflower plants can also be associated with the direct effect of Zn-induced ROS on the enzyme. In the present study, Zn-stressed plants supplemented with SNP, SA, and SNP + SA revealed a significant increase in APX, CAT, and GPX activity in comparison with Zn alone. These results agree well with those of Kazemi et al. (2010), who examined the efficacy of SNP and SA treatment on nickel-stressed canola plants. Wang et al. (2013) also reported that SA and NO are able to increase Fe uptake in ryegrass plants exposed to cadmium stress. It has been shown that SA and NO can perform indirectly in activating antioxidative responses to relieve heavy metal toxicity. It is well known that H^+-ATPase in the plasma membrane performs a noteworthy role in the transport of ions like Fe. There are several studies confirming that SA and NO could raise H^+-ATPase activity and accordingly induce uptake of Fe (Wang et al., 2013). Therefore, the elevated activity of APX, CAT, and GPX in Zn-stressed plants supplemented with SNP, SA, and the combination of these molecules may be due to SNP- and SA-arbitrated increased accessibility of iron in plants. Upon application of SA, SNP, and their combination, the activity of GPX sharply increased, indicating its more important role as compared to CAT and APX. A sharp induction in GPX activity under SA and SNP supplementation may be attributed to the possible role of SA and SNP in regulation of GPX encoding genes. Recently it was reported that SA can induce GPX activity by the regulation of GPX encoding genes (Khan et al., 2015). Furthermore, enhanced activity of GPX may be attributed to the fact that NO can directly or indirectly activate antioxidant enzymes against heavy metal-induced injury (Kazemi et al., 2010).

In this study, proline content increased dramatically in response to Zn treatment. The observed induction in the level of proline in Zn-stressed safflower plants may be due to the important role of proline in ROS detoxification and its ability to inhibit lipid peroxidation (Gill and Tuteja et al., 2010; Sheetal et al., 2016). However, adding SA and SNP caused a sharp reduction in proline content in Zn-stressed safflower plants. Our results are in agreement with those reported by Krantev et al. (2008), Kazemi et al. (2010), and Wang et al. (2013). Based on our results and other reports about the role of SA in the depletion of proline content, this study suggested that partial relief from Zn stress may be one of probable reasons of decreased levels of proline in Zn-stressed SA-supplemented safflower plants. It has been reported that NO supplementation can induce proline degradation by increasing the activity of proline dehydrogenase (Kazemi et al., 2010). Decreased levels of proline have been reported in some heavy metal-stressed plant species supplemented with SNP (Kazemi et al., 2010; Wang et al., 2013). We suggest that NO is probably able to ameliorate Zn-induced injury by the induction of proline degradation. Furthermore, it has been reported that SA may stimulate the induction of NO biosynthesis (Zottini et al., 2007). Therefore, we suggest that the effect of SA on the depletion of proline content may be indirectly related to the stimulation of NO biosynthesis.

In the present study, our results show that Zn treatment can induce α-tocopherol levels in Zn-stressed safflower plants. In our earlier work, we documented that cadmium treatment is also able to provoke α-tocopherol biosynthesis in cadmium-stressed safflower seedlings (Namdjoyan et al., 2012). In the present study, the observed increase in the levels of α-tocopherol may be, at least in part, because of its significant role in Zn detoxification. In the present study, upon adding SA, α-tocopherol levels remarkably increased in the leaves of Zn-treated plants. Supplementation of SNP also increased the level of α-tocopherol in Zn-stressed plants, but not significantly. Earlier, Munné-Bosch et al. (2007) reported that SA can induce α-tocopherol levels in water-stressed *Arabidopsis* plants. These researchers suggested that adjustment of α-tocopherol levels by SA was possibly connected to SA-mediated plant aging. Whereas high levels of Zn have been reported to stimulate the senescence process in plants (Yadav, 2010), we propose that the induction of α-tocopherol levels in SA-supplemented Zn-stressed safflower plants may be partly attributed to Zn-induced senescence.

In this study we showed that Zn treatment remarkably changes the content of PCs in the leaves of safflower plants. The complexes of PC-heavy metal(loid)s have been

shown to be sequestered into a vacuole as the last step of detoxification (Batista et al., 2014). Similar to our results, in response to Zn, phytochelatin biosynthesis and the complex of Zn with PCs have been indicated in *Arabidopsis* (Kühnlenz et al., 2016). There are reports regarding the effect of heavy metals on the upregulation of genes related to PCs biosynthesis (Mishra et al., 2006; Tennstedt et al., 2009). In safflower plants exposed to Zn excess, the induction of PC content can be due to induction of genes responsible for PC biosynthesis, like PC synthase (Mishra et al., 2006), and also it may partly be due to enhanced PC movement from roots through vascular tissues of the plant (Kühnlenz et al., 2016). There was no meaningful difference in the PC content between Zn-stressed and SA + Zn-treated plants. Thus, it seems that SA did not influence the PC content in Zn-stressed safflower plants. On the other hand, our results revealed that the addition of SNP to Zn-treated plants induced a significant increase in the PC content in leaves. Increase in the PC content has been reported in Cu-stressed seedlings of rice exposed to SNP treatment (Mostafa et al., 2014). This probably implies an effective role of SNP in metal detoxification via PC biosynthesis induction. Previously, Elviri et al. (2010) reported that NO, as an identified regulator of protein activation by S-nitrosylation, may raise heavy metal inactivation through the alteration of phytochelatins in cadmium-stressed *Arabidopsis thaliana* plants.

Based on the obtained results, Zn excess induced oxidative damage, which was indicated by the inhibition of growth and chlorophyll content, and increase in oxidative stress markers with changes in enzymatic and nonenzymatic antioxidant responses. It seems likely that SA and SNP play a protective role against oxidative stress induced by Zn excess through the modulation of antioxidant systems. Furthermore, the data showed that, at least in part, the combination of SA and SNP is better able to reduce the deleterious effects of Zn toxicity. An increase in PC content in shoots together with decreased roots to shoot translocation of Zn, as discussed in our previous study (Namdjoyan et al., 2017), may be a beneficial strategy to counteract Zn toxicity in Zn-stressed plants supplemented with SA and SNP.

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