Ecotoxicology
DOI 10.1007/s10646-017-1806-3

Interactive effects of Salicylic acid and nitric oxide in alleviating zinc toxicity of Safflower (*Carthamus tinctorius* L.)

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Accepted: 8 April 2017

Abstract The purpose of this study was to assess the possible protective role of exogenous salicylic acid (SA), sodium nitroprusside (SNP), a donor of nitric oxide, and their combination on 21-day-old safflower (*Carthamus tinctorius* L.) seedlings grown under zinc (Zn) stress. The results revealed that exposure to 500 µM $ZnSO_4.7H_2O$ for 10 days markedly reduced the root and shoot dry weights in Zn-treated plants, while the application of SA, SNP and specially SA + SNP significantly increased the root and shoot dry weights in seedlings subjected to Zn stress. Addition of SA, SNP and SA + SNP interestingly reduced root-to-shoot translocation of zinc and increased significantly the level of glutathione (GSH) and ascorbate (ASC) in leaves of Zn-stressed plants. The Zn-treated plants supplemented with SA and SNP revealed an improved activity of ascorbate-glutathione cycle enzymes and those enzymes which are involved in glyoxalase system as compared to the plants treated with Zn only. However, no significant relationship was found between SA or SNP supplementation and glutathione S-transferase activity in Zn-stressed plants. These findings demonstrate that exogenous application of SA or SNP could ameliorate the negative effects of Zn on safflower plants probably by stimulation of antioxidant defense and glyoxalase systems.

Keywords Antioxidant system · *Carthamus* tinctorius · Nitric oxide · Salicylic acid · Zinc toxicity

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Introduction

Amongst the environmental pollutants, metals are considered as very important and highly toxic pollutants. Zinc is a known micronutrient with important physiological functions in plants. It plays a vital role in the normal growth and development of plants. Moreover, Zn is known to play a critical role in the cell division, protein synthesis and also in many metabolic reactions as enzyme cofactor. At high concentrations, however, Zn has been found to be toxic to plant growth and development by promoting the production of reactive oxygen species (ROS) (Li et al. 2013). Elevated Zn concentration in the soil can be attributed to both natural processes and anthropogenic activities. At the cellular level, excess Zn affects mitotic activity, membrane integrity and permeability, and can even kill cells (Subba et al. 2014).

The production of ROS must be carefully regulated to avoid oxidative damage. Plant cells are normally protected against this oxidative damage by a broad spectrum of antioxidant systems. These antioxidant defense systems mainly include non-enzymatic antioxidant compounds (e.g., GSH and ASC) in addition to antioxidative enzymes (e.g., superoxide dismutase, catalase, ascorbate peroxidase, monodehydro-ascorbate reductase, dehydro-

Published online: 20 April 2017

ascorbatereductase and glutathione reductase) (Gietler et al. 2016; Gill and Tuteja 2010).

Superoxide dismutase (SOD) is a key enzyme responsible for catalyzing dismutation of highly reactive $O_2^{\bullet-}$ to O_2 and H_2O_2. The resulting H_2O_2 is further decomposed to water and oxygen either by ascorbate peroxidase (APX) of the ascorbate-glutathione cycle or by catalase (CAT) localized in the cytoplasm and other cellular compartments. Ascorbate peroxidase simultaneously catalyses the reduction of H_2O_2 and the oxidation of ascorbate with the generation of monodehydroascorbate (MDHA). Monodehydroascorbate reductase (MDHAR) may be critical in maintaining proper ascorbate concentrations by directly reducing MDHA to ascorbate. Dehydro-ascorbatereductase (DHAR) is an important enzyme required in the ascorbate-glutathione cycle in plants. This enzyme can reduce dehydroascorbate (DHA) to ascorbate using GSH (Hasanuzzaman and Fujita 2013; Zhao et al. 2015).

Glutahione, in its reduced and oxidized forms, GSH and GSSG, plays a key role in various environmental stresses. GSH is the major intracellular antioxidant inside the cell and is the precursor of phytochelatins (Gietler et al. 2016).

Glutathione reductase GR is the complementary enzyme of the ascorbate-glutathione cycle that helps in maintaining a high GSH/GSSG ratio, for protection against oxidative damage (Mishra et al. 2011).

Glutathione S-transferases (GSTs), also known as glutathione transferases, are ubiquitous enzymes involved in cellular detoxification and response to the oxidative stress including drought, salt, heavy metals, and so on by catalyzing the nucleophilic conjugation of reduced tripeptide glutathione (GSH; g-Glu-Cys-Gly) into a wide variety of hydrophobic, electrophilic and cytotoxic compounds. Previous studies revealed that excessive ROS induce an increase in GST levels, and then the GSTs participate in rapid clearance of superoxide radicals to protect plant cells from oxidative damage (Hasanuzzaman and Fujita 2013).

Methylglyoxal (MG) is another potent cytotoxic compound which accumulates under abiotic stresses, including heavy metal toxicity. There are several reports showing MG can lead to cell death by oxidation of protein, inactivation of enzymes and DNA damage (Talukdar 2016). In plants, MG detoxified by the maintenance of GSH homeostasis via glyoxalase (Gly) enzymes: while glyoxalase I (GlyI) utilizes GSH to convert MG into its thioester, S-D-lactoylglutathione (SLG) whereas the glyoxalase II (GlyII) hydrolyzes this thioester to regenerate GSH. There are several reports indicating the coordinated inductions both of antioxidant defense system and glyoxalase system to scavenge ROS and MG (Hasanuzzaman and Fujita 2013).

A large number of studies have shown that the exogenous application of some plant signal molecules can alleviate the toxic effects of heavy metals (Dong et al. 2015; Xu et al. 2015). Nitric oxide (NO) is a bioactive gaseous molecule involved in signaling processes within plants that participates in many physiological functions under normal and stress conditions in plant cells. Also, application of the NO donor sodium nitroprusside (SNP) has been shown to be involved in mediation of environmental stresses such as heavy metals. Nitric oxide is itself a reactive nitrogen species and its effects on different types of cells have proved to be either cytoprotective or cytotoxic, depending on its concentration and on the position of action (Kazemi et al. 2010; Zhao et al. 2015).

Salicylic acid (SA), a simple phenolic compound, is another signal molecule contributing to mediate a variety of physiological processes and responses to biotic and abiotic stress (Wang et al. 2013). It is well-established that the exogenous application of SA can ameliorate the heavy metal-induced toxicity. Furthermore, SA alters the activities of antioxidant enzymes and increases plant tolerance to abiotic stresses (Dong et al. 2015; Xu et al. 2015). It has been shown that SA can ameliorate the toxic effects of heavy metal-induced oxidative stress by modulating antioxidant system-components and also through the reduction of membrane lipid peroxidation. SA has been reported to improve plant-abiotic stress tolerance by regulating many aspects in plants at the gene level (Kazemi et al. 2010; Khan et al. 2015).

It has been found that SA can stimulate the synthesis of NO by elevating the activity of NO synthesizing enzymes. A large number of studies have reported that the application of SA and NO partially decreased the deleterious effects of some abiotic stresses, including salinity and heavy metal stress (Khan et al. 2015).

Nowadays, phytoremediation of metals from the environment serves as a cost-effective green technology. Safflower (*Carthamus tinctorius* L.) is an annual crop plant of the Asteraceae family, with the wide geographical distribution. It has long been used for coloring and flavoring the food. Safflower edible oil can be used for industrial purpose, for the preparation of drugs and also for the cosmetics production. Safflower possesses interesting characteristics in terms of heavy metal accumulation. It has been reported that safflower is capable of accumulating high levels of cadmium, lead and zinc in its different parts without showing symptoms of toxicity (Namdjoyan et al. 2011; Srivastava and Bhagyawant 2014; Angelova et al. 2016). Although the heavy metal tolerance of this plant have been previously studied, to our knowledge, there is no study dealing with the protective role of exogenous NO and/or SA against the Zn-induced oxidative stress. Moreover, the interactive effects of NO and SA on antioxidant defense and glyoxalase systems in the expression of Zn toxicity have rarely been assessed. Hence, this investigation aimed

to evaluate the possible effects of SNP (as NO donor) and SA singly and in combination on the antioxidative defense and glyoxalase systems in Zn-stressed safflower plants.

Material and methods

Plant material and growth conditions

The Safflower (*Carthamus tinctorius* L. cv. Arak2811) plant was selected based on previous research showing its ability to tolerate and accumulate high concentrations of heavy metals such as Zn and also its capability to grow in a heavy metal-contaminated soil (Namdjoyan et al. 2011; Angelova et al. 2016). The seeds were surface-sterilized with a 0.1% (w/v) $HgCl_2$ solution for 8 min. After rinsing 4–5 times with sterile distilled water, seeds were germinated under sterile conditions on a wet filter paper at 25 °C for 48 h. Eight germinated seedlings of uniform size were then transferred to plastic pots (volume 500 ml) filled with perlite and watered with full-strength Arnon and Hoagland nutrient solution (Arnon and Hogland 1940). 21-day-old plants were transferred to fresh medium (3 plants per pot) supplemented with $ZnSO_4.7H_2O$ either with or without SA or SNP (NO donor) or SA + SNP. 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 concentration (data not presented). Two Zn levels (0 and 500 µM $ZnSO_4.7H_2O$) and two levels of both SA and SNP (0 and 100 µM) were applied. Details of whole treatments per pots were finally 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 $ZnSO_4.7H_2O$). The treatments were arranged in a completely randomized design with five replicates, for a total of 40 containers. Hoagland solution was adjusted to pH 6.5 and renewed every 2 days. Plants were grown in controlled-environment growth room at 16/8 light/dark photoperiod and photon flux density 200 µmol m^{-2} s^{-1}, Day/night temperature of 26/22 °C and 65 ± 5% relative humidity. After 10 days of growth with the above conditions, the plants were harvested and the roots and shoots were separated and washed with deionized distilled water. For the estimation of plant dry mater and Zn concentration, the plants were dried at 80 °C for 48 h. For the enzyme activity determination, fresh plant material was frozen in liquid nitrogen and stored at −70 °C until use.

Zinc determination

Roots and shoots (including leaves) were separated, dried to constant weight at 80 °C, and then grounded with a mortar and pestle. Fifty milligrams of plant materials were digested in 5 mL of ternary mixture of HNO3: H2SO4: HClO4 in the ratio of 10:1:4 (v/v/v). Zn Concentration was determined using atomic absorption spectrophotometer (Perkin Elmer, Germany).

ASC and DHA analysis

ASC and DHA were determined by a modified method from Hodges et al. (1996). Total ascorbate was determined in a reaction mixture consisting of 100 µL of supernatant, 500 µL of 150 mM KH_2PO_4 buffer containing 5 mM EDTA and 100 µL of 10 mM dithiothreitol (DTT) to reduce DHA to ASC. After 10 min at room temperature, 100 µL of 0.5% (w/v) *N*-ethylmaleimide was added to remove excess DTT. ASC was assayed in a similar manner except that 200 µL of deionized H_2O was substituted for DTT and *N*-ethylmaleimide. The reaction mixtures were incubated at 40 °C for 1 h and quantified spectrophotometrically at 525 nm. DHA was estimated from the difference of total ascorbate and ASC.

Determination of GSH and GSSG contents

GSH and GSSG contents were determined by the recycling method described by Anderson (1985). Fresh root and shoot samples (500 mg) were homogenized in 0.3 mL of 5% sulfosalicylic acid under cold conditions. Absorbance was read for the determination of GSH at 412 nm using a UV-Vis spectrophotometer. GSSG was calculated by subtracting the GSH from the total glutathione concentration. A standard curve was prepared from varying concentrations of reduced glutathione.

Enzyme assays

Plant samples of a known weight (500 mg fresh weight) were homogenized in 1 mL of 50 mM cold potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol at 4 °C. The homogenate was centrifuged at 15000 *g* at 4 °C for 15 min to remove plant debris. The protein contents in the supernatant were determined according to the method of Bradford (1976), with BSA as a standard. The supernatant was used for the following assays of antioxidant enzyme activities.

MDHAR (EC 1.6.5.4) activity was assayed by monitoring the change in absorbance at 340 nm due to NADH oxidation ($E = 6.2\ \mu M^{-1}\ cm^{-1}$) for 4 min in a 1 mL reaction mixture containing 90 mM potassium phosphate buffer (pH 7.0), 0.0125% Triton ×-100, 0.2 mM NADH, 2.5 mM/L ascorbic acid, 0.25 unit ascorbate oxidase and enzyme extract (Hossain et al. 1984). One unit of ascorbate oxidase is defined by the manufacturer (Sigma Chem. Co.) as the amount that causes the oxidation of 1 µM of ascorbate to MDHA per minute.

DHAR (EC 1.8.5.1) activity was determined by measuring the reduction of dehydroascorbate (DHA) at 265 nm for 4 min (Doulis et al. 1997). The reaction mixture consisted of 90 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 5.0 mM reduced glutathione (GSH) and enzyme extract. The reaction was initiated by the addition of 0.2 mM dehydroascorbate ($E = 14\ \mu M^{-1}\ cm^{-1}$).

GR (EC 1.8.1.7) activity was assayed by following the method of Hasanuzzaman et al. (2011) by monitoring a decrease in absorbance at 430 nm caused by NADPH oxidation ($E = 6.22\ \mu M^{-1}\ cm^{-1}$). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 mM oxidized glutathione (GSSG), 0.2 mM NADPH, and the enzyme aliquot.

GST (EC 2.5.1.18) activity was determined by the method of Hossain et al. (2006) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 700 µL. The enzyme reaction was initiated by the addition of CDNB and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of ($E = 9.6\ \mu M^{-1}\ cm^{-1}$).

Gly I (EC 4.4.1.5) assay was carried out according to Hossain and Fujita (2010). Briefly, the assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the activity was calculated at 240 nm for 1 min ($E = 3.37\ \mu M^{-1}\ cm^{-1}$).

Gly II (EC 3.1.2.6) activity was assayed by following the method of Principato et al. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM SLG in a final volume of 1 mL. The reaction was started by the addition of SLG, and the activity was calculated ($E = 13.6\ \mu M^{-1}\ cm^{-1}$).

All spectrophotometeric analyses were conducted at 25 °C with a UV-vis spectrophotometer (Model UV-1601 PC, Shimadzu, Japan).

Statistical analysis

Values in the figures and tables are the mean values standard error (SE) of three independent experiments with five replicates. Statistical assays were carried out by SPSS software (version 17.0). Mean difference comparison between different treatments was done by ANOVA using the Duncan's multiple range test (DMRT) at a 0.05 probability level.

Results

Exposure to Zn treatment caused visible toxicity symptoms including stunted growth, brownish roots and chlorosis on the leaves. However, in the presence of SA or SNP (NO donor) and specifically SA + SNP, the symptoms of Zn toxicity markedly decreased in Zn-treated plants.

Application of Zn resulted in a significant Zn accumulation in both roots and shoots. However, plants tended to contain a higher Zn concentration in roots than in shoots (Table 1). Upon SA, SNP and SA + SNP supplementation, Zn content decreased significantly in shoots, whereas Zn content in roots changed slightly compared to Zn treatment alone (Table 1).

Table 1 Effect of Zn treatment in the presence of salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on dry weight and Zn concentration of safflower roots and shoots

Treatments	Zn Concentration (mg/g dry wt)		Dry weight (mg/ plant)	
	Roots	Shoots	Roots	Shoots
Con	0.0003^{b}	0.0001^{e}	94.31 ± 4.10^{ab}	742.24 ± 17.82^{b}
SNP	0.0003^{b}	0.0001^{e}	88.53 ± 3.37^{b}	765.36 ± 4.87^{ab}
SA	0.0002^{b}	0.0001^{e}	97.06 ± 3.42^{a}	763.71 ± 5.29^{ab}
SNP + SA	0.0003^{b}	0.0001^{e}	102.11 ± 5.95^{a}	771.90 ± 13.25^{a}
Zn	4103.39 ± 129.31^{a}	572.81 ± 26.74^{a}	36.54 ± 1.85^{e}	327.48 ± 22.04^{d}
Zn + SNP	4112.72 ± 85.14^{a}	503.85 ± 26.12^{b}	43.81 ± 2.44^{d}	186.51 ± 9.34^{f}
Zn + SA	4117.43 ± 57.06^{a}	392.63 ± 14.53^{c}	49.78 ± 3.61^{d}	282.21 ± 11.63^{e}
Zn + SNP + SA	4108.66 ± 62.93^{a}	285.27 ± 11.87^{d}	68.06 ± 3.59^{c}	693.17 ± 16.18^{c}

Data are means of three experiments with five replicates. Different letters (a–f) indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

Table 1, shows the effects of Zn treatment alone or together with SA and SNP on biomass production, expressed as dry weight, of safflower roots and shoots. Roots and shoots biomass production was reduced significantly by Zn treatment as compared with the controls. The root dry weight of Zn-treated plants was affected more severely as compared to shoot dry weight. In Zn-stressed plants, upon application of SA, SNP and specially SA + SNP, dry weight of both roots and shoots increased significantly, as compared to Zn treatment alone (Table 1). Compared to treatment with Zn alone, inductions of 46 and 52%, respectively, were observed in root and shoot dry weight of SA + SNP-supplemented Zn-stressed plants.

Safflower plants treated with Zn showed a significant increase in ASC content by 31% ($P \leq 0.05$), as compared to the control plants. However, upon addition of SA, SNP or SA + SNP, ASC content further increased in leaves, as compared to Zn treatment alone (Fig. 1a).

The results revealed that the level of DHA in leaves of Zn-treated plants increased by 61% ($P \leq 0.05$) as compared to the controls (Fig. 1b). In the presence of SA, SNP and SA + SNP, DHA level significantly decreased in Zn-stressed plants. The lowest level of DHA, which was 48% lower than that of the plants treated with Zn only, was noted in SA-treated Zn-stressed plants (Fig. 1b).

In comparison to the control, a 38% reduction in the ASC/DHA ratio was observed with Zn treatment (Fig. 1c). Interestingly, upon addition of SA, SNP and SA + SNP, the DHA/ASC ratio sharply increased as compared to Zn treatments alone (Fig. 1c). The highest DHA/ASC ratio was observed in SA-treated Zn-stressed plants, which was 59% higher than that of control.

As shown in Fig. 2a, the level of GSH increased significantly in leaves of safflower plants treated with 500 µM Zn as compared with the controls. Application of SA, SNP and SA + SNP caused further increase in GSH content of Zn-stressed plants. The maximum GSH content was noted in SNP treated Zn-stressed plants, which was by 17% higher than that of Zn treatment alone (Fig. 2a).

Zn addition was found to significantly increase GSSG levels when compared to the control. However, upon addition of SA, SNP or SA + SNP, GSSG content markedly decreased in leaves, as compared to Zn treatment alone (Fig. 2b).

Compared to the control, an induction of 19% was observed in the GSH/GSSG ratio of plants stressed with Zn alone. Addition of SA, SNP and SA + SNP sharply increased the GSH/GSSG ratio compared to control and Zn treatment alone. The highest GSH/GSSG ratio, 51% higher than that of the control, was noted in SNP treated Zn-stressed plants (Fig. 2c).

The activity of MDHAR and DHAR exhibited a significant increase in the leaves of Zn-treated plants (Fig. 3a, b). Zn caused increases in DHAR and MDHAR activities by 46 and 67%, respectively, in comparison to their respective controls. However, treatment with SA, SNP and SA + SNP resulted in more remarkable increase in the DHAR activity than those treated with Zn alone (Fig. 3b). In contrary, upon application of SA or SNP, specially SA + SNP, the activity of MDHAR was inhibited as compared to Zn treatment alone (Fig. 3a).

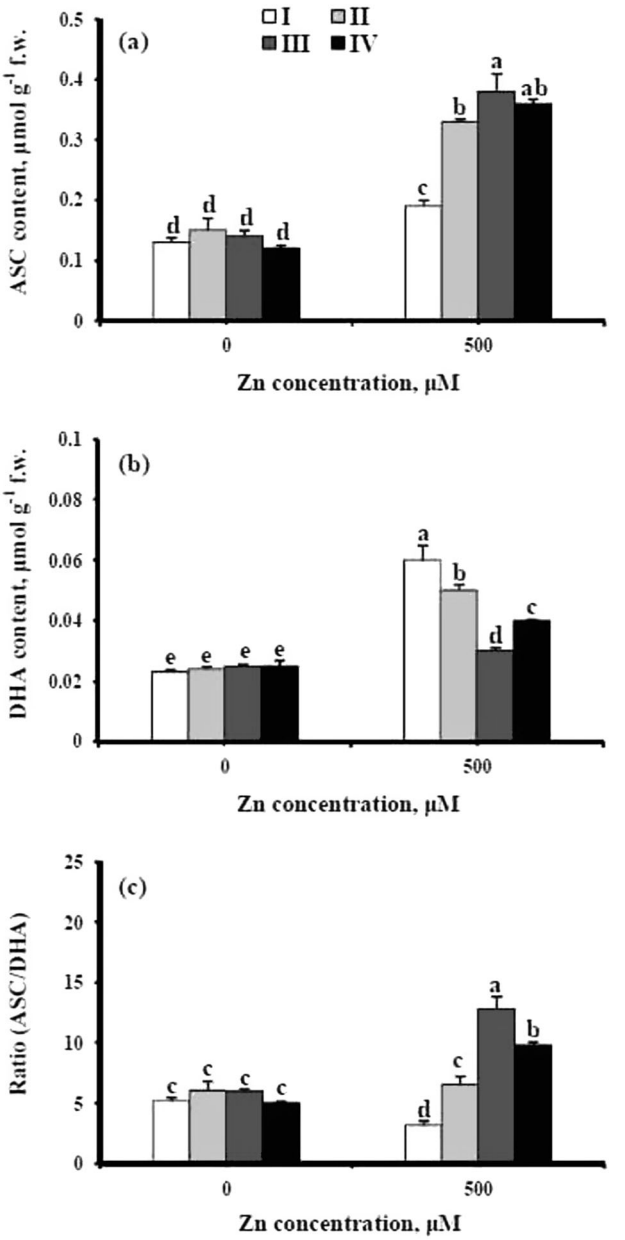

Fig. 1 Effects of Zn in combination with salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on ascorbate (ASC) content **a**, dehydro-ascorbatereductase (DHA) content **b**, and the DHA/ASC ratio **c** in leaves. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Values represent mean ± SE of three experiments with the five replicates. Different letters indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

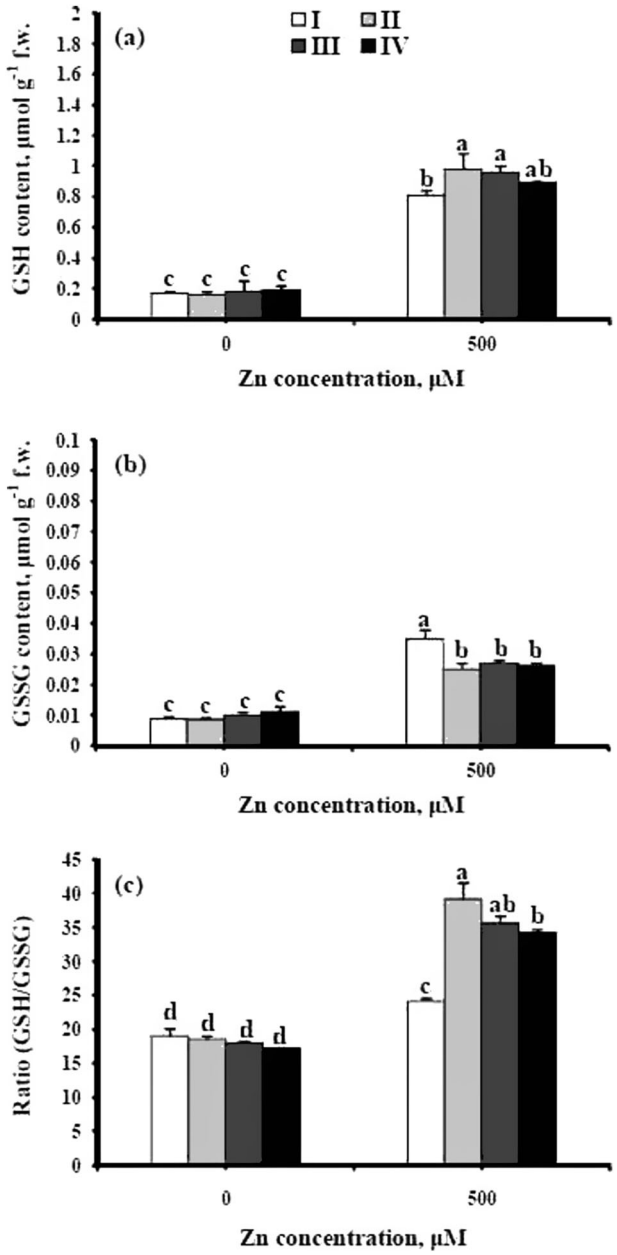

Fig. 2 Effects of Zn in combination with salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on reduced glutathione (GSH) content **a**, oxidized glutathione (GSSG) content **b**, and the GSH/GSSG ratio **c** in leaves. (I) Without SA and SNP, (II) 100 μM SNP, (III) 100 μM SA, and (IV) 100 μM SA + 100 μM SNP. Values represent mean ± SE of three experiments with the five replicates. Different letters indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

GR activity in leaves strongly increased in response to Zn application and the activity of this enzyme was about 3.4 times ($P \leq 0.05$) higher than that of control. However, upon supplementation of SA, SNP and SA + SNP, GR activity sharply decreased in plants treated with Zn only (Fig. 3c).

In comparison to the control, the GST activity in leaves of Zn-treated plants interestingly increased by 39% ($P \leq 0.05$). However, upon supplementation of SA, SNP and SA + SNP the activity of GST slightly increased in Zn-treated plants (Fig. 3d).

As shown in Fig. 4a, b, treatment with Zn alone caused a slight increase in GlyI and GlyII activities in leaves. In contrast, the Zn-treated plants supplemented with SA, SNP and SA + SNP significantly induced the activity of GlyI and GlyII compared to the plants treated with Zn only. The maximum GlyI and GlyII activity was noted in SNP-treated Zn-stressed plants, which were by 57 and 70% higher, respectively, than their respective controls (Fig. 4a, b).

Discussion

Some visual Zn toxicity symptoms such as chlorosis on leaves and browned root tips were observed in safflower plants treated with Zn alone. Furthermore, exposure to Zn caused a significant decrease in dry biomass of Zn-stressed plants. These results are in accordance with the results of Li et al. (2013), who reported that Zn excess reduced the biomass production of wheat plants. A significant decrease in plant biomass suggested Zn-induce toxicity in safflower plants. We suggested that Zn-induced chlorosis in plant exposed to Zn may be attributed to Fe deficiency, inhibition of chlorophyll synthesis or the degeneration of chlorophyll through an increase in the rate of chlorophylase activity (Kazemi et al. 2010).

In this study, the accumulation of Zn in roots of Zn-treated plants was significantly higher than those in shoots. Interestingly, adding SNP, SA, or SNP + SA to Zn-stressed plants resulted in a substantive decrease in shoot Zn content, while no significant differences were seen in root Zn content between Zn-treated plants and SNP-, SA, or SNP + SA-supplemented Zn-stressed plant. These results indicated that NO and SA application possibly reduced the root to shoot translocation of Zn, hence, causing a low Zn content in the aboveground parts. Consistently, Kazemi et al. (2010) reported that NO and SA could reduce the root to shoot translocation of nickel in nickel-stressed canola plants.

Toxic effect of Zn on biochemistry and physiological processes was evident from reduced biomass in Zn-stressed plants. Roots are the first site of exposure and toxicity to Zn; thus, root biomass was severely affected. Supplementation of SNP, SA and specially SNP + SA, markedly increased the dry weight of Zn-treated plants. Both NO and SA have been reported to ameliorate heavy metal toxicity on growth. NO and SA may effectively reduce the level of ROS generated during stress and also they are able to stimulate the photosynthetic machinery. Thus, they can alleviate ROS-induced growth inhibition during heavy metal stress (Wang et al. 2013).

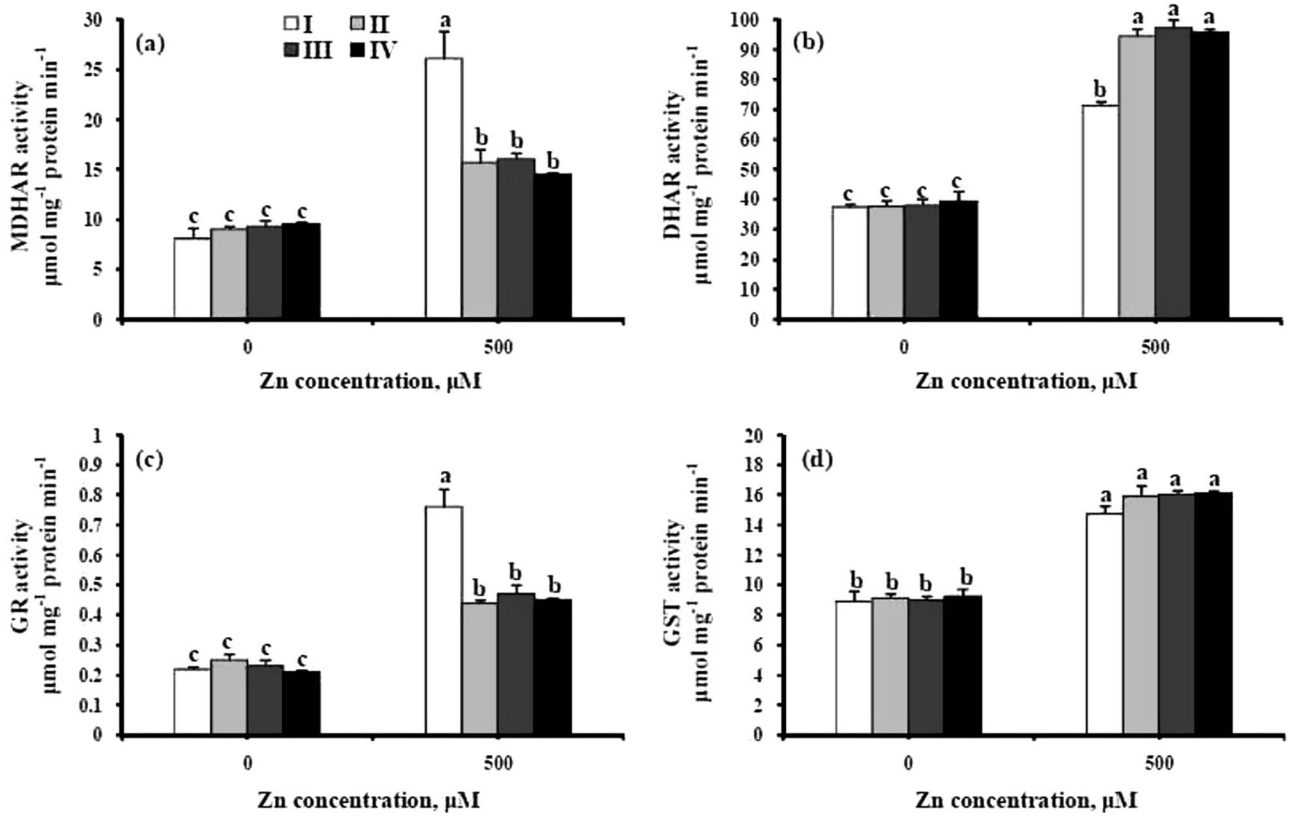

Fig. 3 Effects of Zn in combination with salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on monodehydro-ascorbate reductase (MDHAR) activity **a**, dehydro-ascorbatereductase (DHAR) activity **b**, Glutathione reductase (GR) activity **c**, and Glutathione S-transferases (GST) 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. Values represent mean ± SE of three experiments with the five replicates. Different letters indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

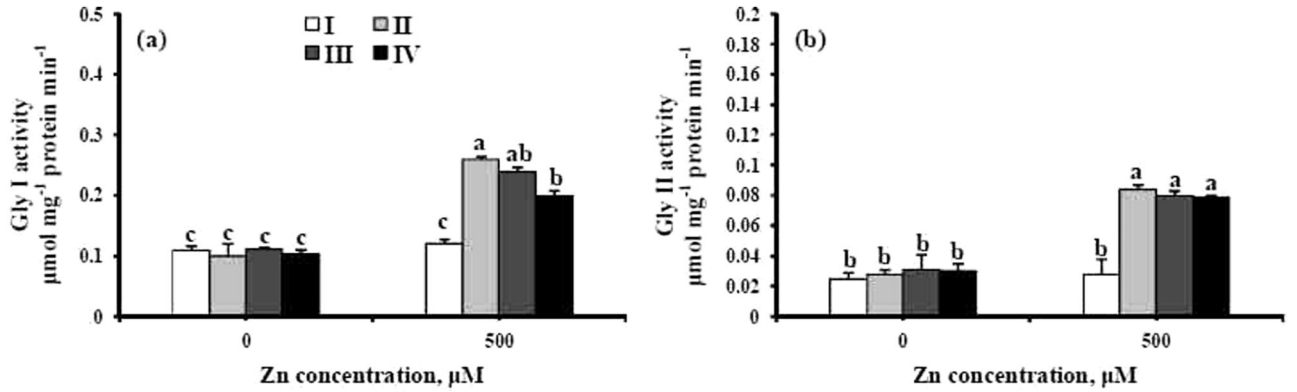

Fig. 4 Effects of Zn in combination with salicylic acid (SA) and sodium nitroprusside (SNP), a donor of nitric oxide, on glyoxalase I (Gly I) **a** and glyoxalase II (Gly II) **b** activity in leaves. (I) Without SA and SNP, (II) 100 µM SNP, (III) 100 µM SA, and (IV) 100 µM SA + 100 µM SNP. Values represent mean ± SE of three experiments with the five replicates. Different letters indicate significant difference between treatments at $P \leq 0.05$, according to Duncan's multiple range test

Ascorbic acid, a powerful water-soluble antioxidant plays a vital role in detoxifying ROS directly or through enzymes restricting oxidative stress. In the present study, a significant increase in the level of ASC was observed in Zn-stressed plants, suggesting a possible defensive role of ASC in Zn-induced oxidative stress. This was consistent with studies in mandarin orange exposed to Zn stress (Subba et al. 2014). The increased level of ASC could be explained by enhanced ROS production and thus activation of H_2O_2-scavenging enzymes of the ascorbate-glutathione cycle.

Accordingly, as discussed below, these H_2O_2-scavenging enzymes showed marked increases in leaves of safflower plants in response to Zn supply. However, the content of ASC and ASC/DHA ratio further increased upon SA and SNP supplementation in Zn-stressed plants, indicating that SA and SNP could possibly led to a more efficient detoxification of Zn through the increased synthesis of non-enzymatic antioxidants such as ASC and also via maintaining a relative higher ratio of ASC/DHA.

In our experiment, GSH content of Zn-stressed plants was substantially higher than the values recorded for control plants. Previous plant studies (Gill and Tuteja 2010) indicated marked increase in GSH content in response to heavy metal which was in agreement with our present study where the content of GSH significantly increased in Zn-stressed plants. Elevated levels of GSH in Zn-stressed plants may be associated with its active role in detoxification of ROS. It has been suggested that enhanced GSH synthesis might be partly attributed to induced transcription of the gene responsible for this process, such as glutathione synthetase and glutathione reductase (Mishra et al. 2011).

Interestingly, GSH content and GSH/GSSG ratio in SNP-, SA-, and SNP + SA- supplemented Zn-stressed plants was markedly higher than in control and plants stressed with Zn alone. Consistently, SNP and SA were reported to stimulate GSH synthesis and also to increase GSH/GSSG ratio in heavy metal-stressed plants (Hasanuzzaman and Fujita 2013; Mostafa and Fujita 2014; Xu et al. 2015). Enhanced GSH content and higher GSH/GSSG ratio in SNP- and SA-supplemented Zn-stressed plants reflects the vital role of these signaling molecules in GSH synthesis and also their important role towards redox regulation.

In the present study, the activity of DHAR and MDHAR substantially increased in Zn-stressed plants. In accordance with our results, Mostafa and Fujita 2014 found that the activity of MDHAR and DHAR was increased by cooper stress in rice seedlings. During detoxification of H_2O_2 by APX in ascorbate-glutathione cycle, MDHA that is produced from ASC oxidation can be reduced by MDHAR or be spontaneously converted to ASC and DHA. DHA is then reduced to ASC by DHAR in a reaction requiring GSH. The activity of DHAR permits plant cells to recycle DHA, and helping to recapture ASC before it is lost. Generally, the accumulation of oxidized ascorbate (DHA) is considered as a harmful factor to the plant cell. Therefore, the function of MDHAR is to limit the formation of MDHA radicals and a higher MDHAR/DHAR activity found in our experiment may be a mechanism for inhibiting MDHA disproportion as well as efficient conversion of DHA to the reduced ascorbate. Interestingly, Zn-treated plants supplemented with SNP, SA and especially SNP + SA further increased the activities of MDHAR and DHAR compared to control and Zn alone. This phenomena might be due to important role of NO and SA to maintain higher substrate concentration for APX. Similar to our results Tewari et al. (2009) reported a strong correlation between the ability of SA and NO to improve abiotic stress tolerance in various plant species via enzymes involved in ascorbate-glutathione cycle such as MDHAR and DHAR.

GR, which plays an essential role in defense system against ROS, increased significantly in Zn-stressed safflower plants. Elevated GR activity in response to heavy metal stress has been reported in several plant species (Li et al. 2013; Mishra et al. 2011). Increased GR activity in this work may be due to enhanced ROS production and thus the activation of H_2O_2-scavenging enzymes of the ascorbate-glutathione cycle such as GR. Our results showed that Zn-stressed plants supplemented with SA did not show any differences in GR activity compared to Zn stress alone, while upon SNP supplementation there was a significant increase in GR activity in Zn-stressed plants. This may be attributed to the role of NO for maintaining higher GSH level and GSH/GSSG ratio in Zn-stressed plants. NO-induced increase in GR activity under arsenic stress in wheat seedlings has been reported earlier (Hasanuzzaman and Fujita 2013; Mostafa and Fujita 2014).

In the current study, the activity of GST, an important group of enzymes involved in heavy metal detoxification, significantly increased in Zn-stressed plants. Similar results of increasing GST activity have been demonstrated in different heavy metal-stressed plants (Gill and Tuteja 2010; Hasanuzzaman and Fujita 2013). Enhanced activity of GST in Zn-treated safflower plants may be due to possible defensive role of this enzyme in Zn induced oxidative stress. However, upon SNP, SA or SNP + SA supplementation there was not a significant increase in GST activity in Zn-stressed plants indicating these molecules did not probably affect GST activity in Zn-stressed plants.

Methylglyoxal is a cytotoxic compound generally formed under environmental stresses including heavy metals. This toxic compound can produce ROS and stimulate oxidative stress. Gly I and Gly II are two major enzymes in glyoxalase system and play a vital role in MG detoxification. Additionally, glyoxalase system also contributes to the redox homeostasis by regenerating GSH (Hasanuzzaman and Fujita 2013; Talukdar 2016). In the present study, an increase in the activities of Gly I and Gly II was observed upon Zn treatment. However, the activities of glyoxalase enzymes in Zn-treated plants changed slightly, confirming that glyoxalase system may not correlate to detoxification of Zn-induced MG. Upon supplementation of Zn-treatment solution with SNP, SA, or SNP + SA, there was a remarkable increase in the activities of Gly I and Gly II in Zn-stressed plants. Similar to our observations Hasanuzzaman and Fujita (2013) observed an

increase in Gly I and Gly II activities in HM-stressed plants. So, it could be speculated that the enhanced glyoxalase enzymes activities in SNP-, SA- supplemented Zn-stressed plants may serve as a possible mechanism for MG detoxification. Since GSH is recycled by the glyoxalase system and also the elevated level of GSH and high GSH/GSSG ratio have shown to increase Gly I and Gly II activities in some HM-stressed plants (Hasanuzzaman and Fujita 2013), it can be supposed that induction in their activities in Zn-stressed plants may result from increased level of GSH and high GSH/GSSG ratio in SNP- and SA-supplemented Zn-treated safflower plants. Accordingly, as discussed, GSH level and GSH/GSSG ratio showed marked increase in Zn-stressed plants in response to the SNP and SA supply (Fig. 2a, b, c).

It can be concluded that, although safflower plant has a high ability to take up and accumulate Zn, the excess Zn can cause injury to the plant, as observed from the severity of leaf symptoms and reduced biomass production. The results of this study indicate that application of exogenous SA and SNP led to considerable reduction in Zn toxicity. Based on our results, apparently, the strategy of tolerance to Zn toxicity in SNP- and SA-supplemented Zn-stressed safflower plants is dependent on the coordinated induction of antioxidant defense and glyoxalase systems. This was evident by the high increase in the activity of the enzymes of ascorbate-glutathione cycle and glyoxalase pathway enzymes along with stronger accumulation of other antioxidant compounds (e.g., GSH and ASC). Moreover, alleviated Zn-induced toxicity in plants supplemented with SA and SNP, may be partly due to inhibition of Zn translocation from roots to shoots or sequestration of Zn in roots. In this study, Zn-treated plants showed a great capacity to absorb and accumulate Zn; however, high Zn concentration resulted in oxidative damage in Zn-treated plants. Addition of SA or SNP in particular their combination, decreased the adverse effects of Zn excess. There was a significant difference in root Zn content between control and SA-, SNP- and SA + SNP-supplemented Zn-stressed plants, therefore, SA and SNP probably affected Zn uptake by roots. The data also showed that SA and SNP decreased the root-to-shoot translocation of Zn. Hence we proposed that the phytoremediation potential of Zn-contaminated soils can be enhanced by the coapplication of SA and SNP.

Acknowledgements This work was supported by the funding of Research Council of the Shahr-e-Qods Branch of Islamic Azad University (448/95/DP). Special acknowledgements are given to the editors and reviewers. Also we specially thank Dr. Hamid Mozafari, for invaluable facilities and support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with animal.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548–554

Angelova VR, Perifanova-Nemska MN, Uzunova GP, Kolentsova EN (2016) Accumulation of Heavy Metals in Safflower(*Carthamus tinctorius* L.). Internat J Biol, Biomol, Agricul, Food Biotechnol Eng 10(7):410–415

Arnon DI, Hogland DR (1940) Crop production in artificial solutions and in soils with special references to factors influencing yields and absorption of inorganic nutrients. Soil Sci 50:463–484

Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Dong YJ, Wang ZL, Zhang JW, Liu S, He ZL, He MR (2015) Interaction effects of nitric oxide and salicylic acid in alleviating salt stress of *Gossypium hirsutum* L. J Soil Sci Plant Nut 15:561–573

Doulis AG, Debian N, Kingston-Smith AH, Foyer CH (1997) Differential localization of antioxidants in maize. Plant Physiol 114:1031–1037

Gietler M, Nykiel M, Zagdan´ska BM (2016) Changes in the reduction state of ascorbate and glutathione, protein oxidation and hydrolysis leading to the development of dehydration intolerance in Triticum aestivum L. seedlings. Plant Growth Regul 79:287–297

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physio. Biochemistry 48:909–930

Hasanuzzaman M, Fujita M (2013) Exogenous sodium nitroprusside alleviates arsenic-induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by enhancing antioxidant defense and glyoxalase system. Ecotoxicology 22:584–596

Hasanuzzaman M, Hossain MA, Fujita M (2011) Selenium-induced up-regulation of the antioxidant defense and methylglyoxal detoxification system reduces salinity-induced damage in rapeseed seedlings. Biol Trace Elem Res 143:1704–1721

Hodges DM, Andrews CJ, Johnson DA, Hamilton RI (1996) Antioxidant compound responses to chilling stress in differentially sensitive inbred maize 23 lines. Physiol Plantarum 98:685–692

Hossain MA, Fujita M (2010) Evidence for a role of exogenous glycinebetaine and proline in antioxidant defense and methylglyoxal detoxification systems in mung bean seedlings under salt stress. Physiol Mol Biol Plants 16:19–29

Hossain MA, Nakano Y, Asada K (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physio 25:385–395

Hossain MZ, Hossain MD, Fujita M (2006) Induction of pumpkin glutathione *S*-transferase by different stresses and its possible mechanisms. Biol Plantarum 50:210–218

Kazemi N, Khavari-Nejad RA, Fahimi H, Saadatmand S, Nejad-Sattari T (2010) Effect of exogenous salicylic acid and nitric oxide on lipid peroxidation and antioxidant enzyme activities in leaves of *Brassica napus* L. under nickel stress. Sci. Hortic 126:402–407

Khan MIR, Fatma M, Per TS, Anjum NA, Khan NA (2015) Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. Front Plant Sci 6:462. doi:10.3389/fpls.2015.00462. PMID: 4485163

Li X, Yang Y, Jia L, Chen H, Wei X (2013) Zinc-induced oxidative damage, antioxidant enzyme response and proline metabolism in roots and leaves of wheat plants. Ecotoxicol Environ Saf 89:150–157

Mishra S, Jha AB, Dubey RS (2011) Arsenite treatment induces oxidative stress, upregulates antioxidant system, and causes phytochelatin synthesis in rice seedlings. Protoplasma 248: 565–577

Mostofa MG, Fujita M (2014) Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. Ecotoxicology 22:959–973

Namdjoyan SH, Khavari-Nejad RA, Bernard F, Nejad-Sattari T, Shaker H (2011) Antioxidant defense mechanisms in response to cadmium treatments in two safflower cultivars. Russ J Plant Physiol 58:403–413

Principato GB, Rosi G, Talesa V, Giovannini E, Uotila L (1987) Purification and characterization of two forms of glyoxalase II from the liver and brain of Wistar rats. Biochem. Biophys Acta 911:349–55

Srivastava N, Bhagyawant SS (2014) In vitro accumulation of lead nitrate in safflower seedling and its impact on plant protein. Plant Knowledge. Journal 3:39–46

Subba P, Mukhopadhyay M, Mahato SK, Bhutia KD, Mondal TK, Ghosh SK (2014) Zinc stress induces physiological, ultra-structural and biochemical changes in mandarin orange (Citrus reticulata Blanco) seedlings. Physiol Mol Biol Plants 20:461–473

Talukdar D (2016) Exogenous thiourea modulates antioxidant defense and glyoxalase systems in lentil genotypes under arsenic stress. J Plant Stress Physiol 2:9–21

Tewari RK, Kumar P, Kim S, Hahn EJ, Paek KY (2009) Nitric oxide retards xanthine oxidase-mediated superoxide anion generation in Phalaenopsis flower: an implication of NO in the senescence and oxidative stress regulation. Plant Cell Rep 28:267–279

Wang Q, Liang X, Dong Y, Xu L, Zhang X, Kong J, Liu S (2013) Effects of exogenous salicylic acid and nitric oxide on physiological characteristics of perennial ryegrass under cadmium stress. Plant Growth Regul 32:721–731

Xu LL, Fan ZY, Dong YJ, Kong J, Bai XY (2015) Effects of exogenous salicylic acid and nitric oxide on physiological characteristics of two peanut cultivars under cadmium stress. Biol. Plant 59:171–182

Zhao H, Jin Q, Wang Y, Chu L, Li X, Xu Y (2015) Effects of nitric oxide on alleviating cadmium stress in Typha angustifolia. Plant Growth Regul 78:243–251