PROTECTIVE ROLE OF EXOGENOUS NITRIC OXIDE AGAINST ZINC TOXICITY IN *PLANTAGO MAJOR* L.

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Abstract. Zinc is one of the heavy metal in plants that causes toxicity at high concentration via producing Reactive oxygen species. Nitric oxide can protect cells from oxidative stress produced by reactive oxygen Species. Effect of different concentrations of Zn (0, 100, 300 and 500 μM) and interaction with sodium nitroprusside (SNP, a donor of NO) (100 and 200 μM) were studied on growth parameter (total dry Weight) and some physiological factors (chlorophyll a, chlorophyll b, hydrogen peroxide and malondialdehyde contents), antioxidant enzymes (catalase, peroxidase, ascorbate peroxidase and Superoxide dismutase) in *Plantago major* L. Excess Zn reduced dry weight and chlorophyll content, resulting a decrease in photosynthesis. Zn stress induced the production of hydrogen peroxide (H_2O_2), leading to malondialdehyde (MDA) accumulation. Furthermore, it was found that the activities of antioxidant enzymes in Zn-treated plants such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) were decreased but peroxidase (POD) and catalase (CAT) were increased. The use of NO especially in low concentration reversed Zn-induced negative effects whereas high concentration of NO had no obvious alleviating effect on Zn toxicity in *Plantago major* L. In particular, application of 100 μM SNP could mitigate Zn stress, as a defense mechanism of the plant against Zn toxicity.
Keywords: *heavy metal stress, sodium nitroprusside, antioxidative enzymes, ROS, hydrogen peroxide*

Introduction

Zinc is an essential nutrient element for plants, as it is the component of many proteins like Zn finger-containing transcription factors, carbonic anhydrase, Cu/Zn superoxide dismutase and Zn metalloproteases (Broadley et al., 2007). Many of these proteins have critical roles in the uptake and transport of Zn, transcriptional regulation, RNA binding, regulation of apoptosis and protein–protein interactions (Ciftci-Yilmaz and Mittler, 2008). Zn toxicity can result in nutrient imbalance and induce the generation of excess reactive Oxygen species (ROS) (Wang et al., 2009). The excessive formation of ROS can oxidize various cellular components, resulting in lipid peroxidation, membrane leakage, and enzyme inactivation, which can finally lead to oxidative injury and alteration in the cell structure (Romero-Puertas et al., 2007).

Plants have many detoxification and tolerance mechanisms that mitigate and repair the ROS damages. One of these mechanisms includes antioxidant systems, which help them, survive in the altered environment. Antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), Catalase (CAT) and guaiacol peroxidase (POD), as well as

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antioxidant concentrations, like ascorbic acid, are activated by plants to alleviate oxidative stress (Tewari et al., 2008).

NO is an essential signaling molecule which modulates plant resistance to various abiotic stresses (Yu et al., 2014). NO is a highly reactive, membrane-permeable free radical that has recently emerged as a very important signaling molecule and antioxidant which triggers several types of redox-regulated (defense-related) gene expressions, directly or indirectly, to establish plant stress tolerance (Sung and Hong, 2010). The application of an NO donor, SNP, confers tolerance to various abiotic stresses in plants by enhancing their antioxidant defense system (Xu et al., 2010).

Several lines of study have shown that the protective effect of NO against abiotic stress is closely related to the NO-mediated reduction of ROS in plants (Hasanuzzaman et al., 2010). Exogenous application of SNP enhances plant tolerance to heavy metals (Oliviera et al., 2016) and oxidative stress (Esim and Atici, 2013). A recent report showed that NO was associated with long-term Zn tolerance in *Solanum nigrum* (Xu et al., 2010). NO involved on detoxification of H_2O_2 via modulation of antioxidant enzymes such as catalase (CAT),peroxidase (POD) and ascorbate peroxidase (AXP) and keeping of cell redox couple and non-protein antioxidant including thiol, ascorbate (Tewari et al., 2008). It also has been evidenced that NO stimulated the biosynthetic pathway of glutathione (GSH) in plant cells with an enhanced tolerance against oxidative stress (Xiong et al., 2010).

Plantago major is a medicinal herb with potent antioxidant effect .It belongs to the family of Plantaginaceae and is widely found in Europe and Asia in which Malaysia is one of the countries of its Habitat (Beara et al., 2009).

Nevertheless, the influence of exogenous NO on Zn stress tolerance in *Plantago major* plants is not yet fully understood. The present study was carried out to assess the impact of exogenous NO on reducing Zn toxicity in *Plantago major* L.

Materials and Methods

Plant material and culture conditions

Plantago major L. seeds were sterilized with 5% sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated on moist filter paper in the dark at 27°C for one week. Then, seedlings of uniform size were transferred to plastic pots filled with perlite (2 plants per pot) and irrigated by distilled water for 10 days. The seedlings were then nourished with half-strange Hoagland solution for three weeks, during 3 leaves stage irrigation was continued with half-strange Hoagland solutions containing different concentrations of ZnSO4 (0, 100, 300 and 500 μM) and sodium nitroproside (0, 100 and 200 μM) alone and together for two weeks. The experiment was carried out under a controlled environment, day/night temperature of 27/18°C and 65 ± 5% relative humidity. For the estimation of plant dry matter content, the plants were dried at 80°C for 48 h, to give a constant weight.

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Determination of photosynthetic pigments

The photosynthetic pigments (chlorophyll a and b) was determined as per the method of Lichtenthaler (1987). Chlorophyll of experimental plant was extracted with 80% acetone and centrifuged. Supernatant was taken and optical density was measured at 663 nm, 645nm. The chlorophyll a and b was calculated by Eq.1 and 2.

$$\text{Chlorophyll a } (\mu g/ml) = 12.21 \times A663 - 2.81 \times A645 \quad \text{(Eq. 1)}$$

$$\text{Chlorophyll b } (\mu g/ml) = 20.13 \times A645 - 5.03 \times A663 \quad \text{(Eq. 2)}$$

where A is the observed OD.

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring MDA, a major thiobarbituric acid reactive species (TBARS), and product of lipid peroxidation (Heath and Packer 1968). Samples (0.2 gr) are ground in 3 mL of trichloroacetic acid (0.1%, w/v). The homogenate was centrifuged at 10,000 g for 10 min and 1 mL of the supernatant fraction was mixed with 4 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 min, chilled on ice, and then centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

Estimation of hydrogen peroxide (H_2O_2)

H_2O_2 concentration from leaf samples was measured according to the procedure of Velikova et al. (2000). Fresh leaf tissue (0.5 gr) was homogenized with 5 mL of 0.1% (w/v) (TCA) in a pre-chilled mortar and pestle and the homogenate was then centrifuged at 12,000 g for 15 min. To 0.5 mL of the Supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide (1 M) were added. The mixture was vortexed and its absorbance was read spectrophotometrically at 390 nm.

Determination of antioxidant enzymes

For extraction of antioxidative enzymes, leaves and roots were homogenized with 50 mM Na2HPO4-NaH2PO4 buffer (pH 7.8) containing 0.2 mM ethylene diamine tetra acetic acid (EDTA) and 2% insoluble polyvinylpyrrolidone in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulted supernatant was used for the determination of enzyme activities. The whole extraction procedure was carried out at 4°C. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 according to the method of Patra et al. (1978). POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and

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Cunningham, 1969). APX activity was measured by the decrease in absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981).

Statistical analysis

All data presented here are the mean values of three independent experiments with three replicates. All results were analyzed statistically by two-way ANOVA with SAS 9.1.3 software and means were compared with the LSD test ($P < 0.05$).

Results

Biomass

Effect of different concentrations of Zn (0, 100, 300 and 500 μM) and interaction with sodium nitroprusside (100 and 200 μM) on plant growth, expressed as the total dry weight shown in *Table 1*. Zn exposure inhibited the growth of *Plantago major* significantly compared with control (Zn0Snp0), however, this inhibition was alleviated by the additions of 100 μM SNP. When applying 200 μM SNP into Zn-treated solution, Zn-induced inhibition on plant growth was not mitigated and the total dry weight reduced (*Table 1*).

Photosynthetic pigments (chlorophyll a and b)

A significant decrease in chlorophyll a and b contents was observed in the leaves of *Plantago major*, which were exposed to Zn stress. Compared with Zn-stressed plants, the addition of 100 μM SNP alleviated Zn toxicity in the photosynthetic apparatus. Whereas high concentration of SNP (200 μM) had no alleviated effects on the Zn-decreased chlorophyll contents (*Table 1*).

Hydrogen peroxide content

H_2O_2 content in leaf and roots of *Plantago major* was increased depending on Zn concentrations. Under SNP concentrations, H_2O_2 content was decreased in low concentration and increased in high concentration of SNP. However, interactions between Zn and SNP concentrations showed that under Zn concentrations, the additions of SNP 100μM inhibited Zn-induced H_2O_2 generation significantly (*Table 1*). This result indicates that the accumulation of H_2O_2 can reflect the oxidative stress and the changes of antioxidant in plants and indicates that low concentration of NO acts as an efficient ROS scavenger.

Malondialdehyde content

In the present study, the concentration of MDA was significantly increased ($P<0.05$) by Zn treatments in leaf and roots, which indicated an enhanced level of lipid peroxidation in metal-exposed plants. Under SNP concentrations, MDA content increases with increasing SNP concentrations. The application of SNP to Zn treatments shows that the low concentration of SNP (100 μM) can alleviate the effects of Zn stress on lipid peroxidation (*Table 1*).

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Table 1. *Effects of different concentrations of SNP on total dry weight, Chl a and b, Leaf and Root MDA content, Leaf and Root H_2O_2 content in Plantago major under Zn stress*

Zn	SNP	Total dry weight (gr)	Chl a (mg/g FW)	Chl b (mg/g FW)	Leaf MDA content (nmol. g^{-1}.FW)	Root MDA content (nmol. g^{-1}.FW)	Leaf H_2O_2 content (µmol. g^{-1}.FW)	Root H_2O_2 content (µmol. g^{-1}.FW)
0	0	0.743 a	2.767 ab	0.76067 a	26.5 j	17.563 j	22.737 i	17.46 g
	100	0.71 b	2.837 a	0.7623 a	27.417 i	17.993 j	23.073 i	17.267 g
	200	0.63 c	2.567 bcd	0.727 b	33.657 g	21.444 i	24.93 h	19.733 f
100	0	0.69 b	2.433 de	0.7273 b	34.727 f	25.163 h	26.956 g	22.123 e
	100	0.75 a	2.673 abc	0.7723 a	30.646 h	21.96 i	25.213 h	21.903 e
	200	0.633 c	2.313 e	0.7177 b	36.33 de	26.693 g	27.393 f	23.863 d
300	0	0.6 d	2.02 f	0.6206 d	36.123 e	32.327 e	28.507 e	23.766 d
	100	0.687 b	2.47 cde	0.687 c	33.35 g	28.587 f	26.83 g	21.833 e
	200	0.567 e	1.753 g	0.5943 e	37.016 d	34.373 c	29.703 d	24.977 c
500	0	0.47 f	1.45 h	0.462 g	40.203 b	37.7533 b	32.863 b	25.583 b
	100	0.55 e	2.02 f	0.5417 f	38.513 c	33.62 d	30.326 c	23.516 d
	200	0.43 g	1.24 i	0.4126 h	43.97 a	41.18 a	34.416 a	26.326 a

Means followed by the same letter are not significantly different ($P < 0.05$).

Peroxidase enzyme activity

According to *Figs. 1* and *2*, leaf and root POD enzyme activity increased significantly (at 0.05 level) under Zn stress condition. Under SNP concentration enzyme activity increased significantly (at 0.05 level) in 100 µM and decreased significantly (at 0.05 level) in 200 µM. Interaction between Zn and SNP shows that enzyme activity increased significantly in Zn+SNP100 µM and decreased significantly in Zn+SNP200 µM. It seems that NO in low concentration can improve negative effects of Zn stress (*Figs. 1* and *2*).

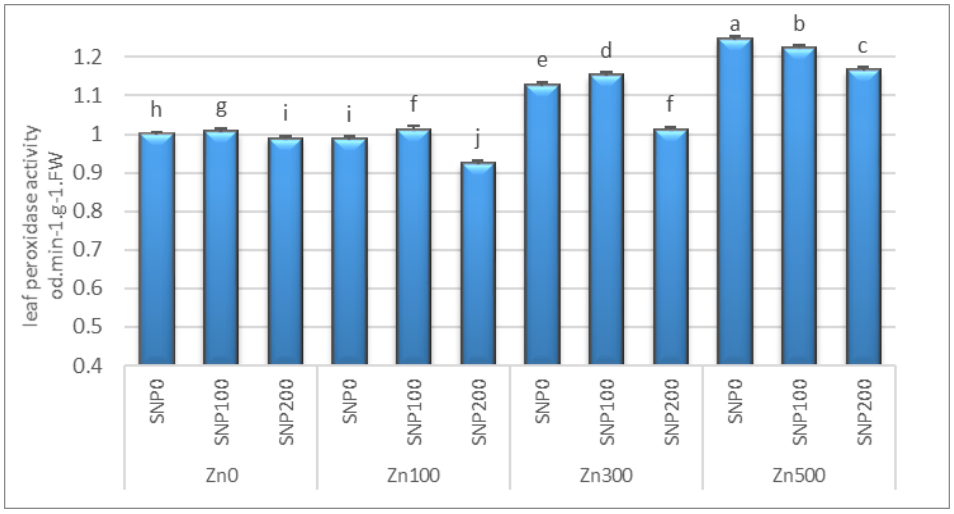

Figure 1. *Effects of different concentrations of SNP on peroxidase activity in leaf of Plantago major under Zn stress (P<0.05)*

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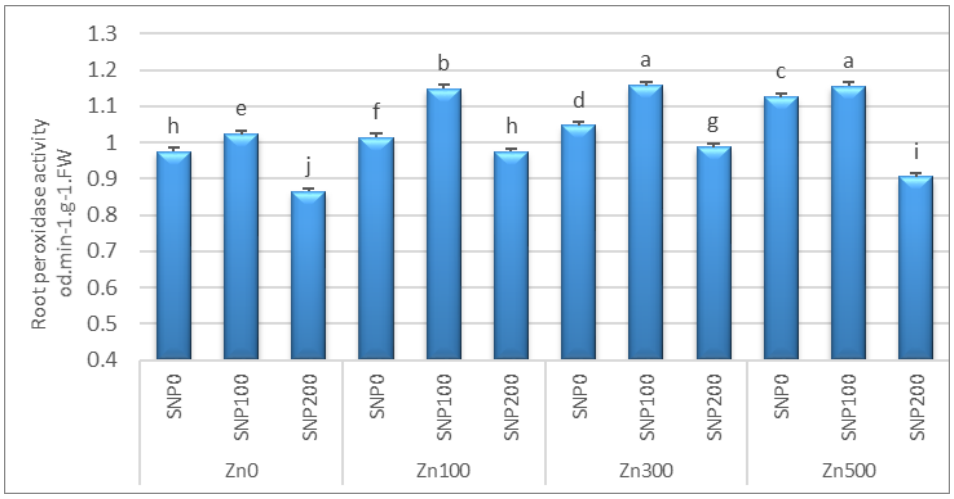

Figure 2. *Effects of different concentrations of SNP on peroxidase activity in root of Plantago major under Zn stress (P<0.05)*

Catalase enzyme activity

CAT enzyme activity increased significantly (at 0.05 level) under Zn stress condition compared with the control plant .Under SNP condition, there was decrease in SNP200 compared with SNP100. Generally, Zn treatment induced the activities of CAT in shoot and root of *plantago major* but this increase was found reversed in 200 µM SNP treatment (*Figs. 3* and *4*).

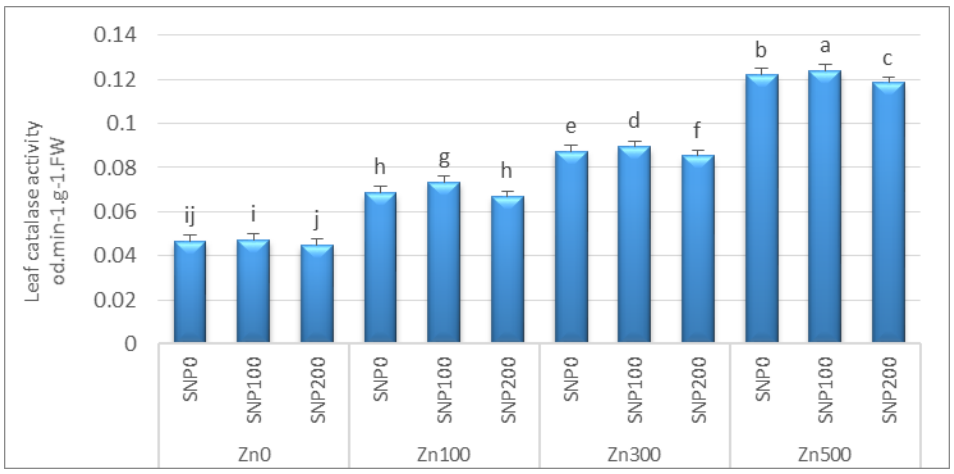

Figure 3. *Effects of different concentrations of SNP on catalase activity in leaf of Plantago major under Zn stress (P<0.05)*

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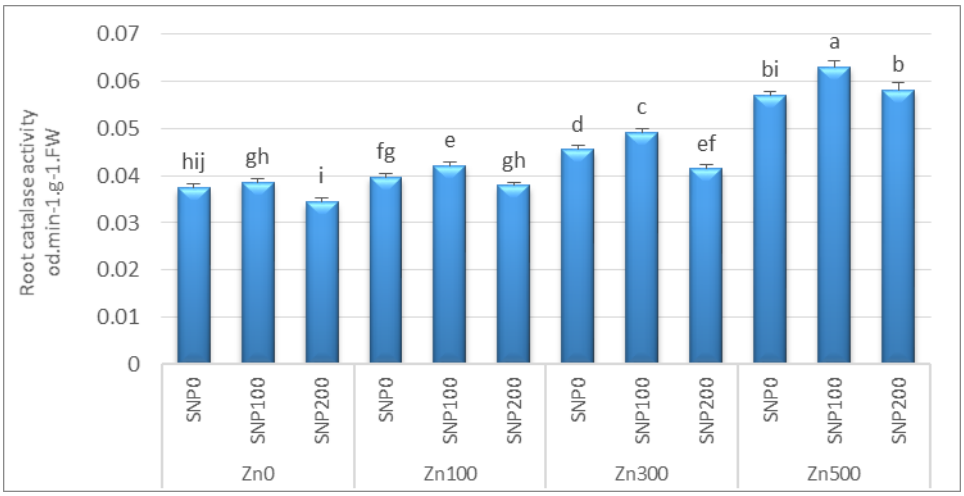

Figure 4. *Effects of different concentrations of SNP on catalase activity in root of Plantago major under Zn stress*

Ascorbate peroxidase enzyme activity

Leaf APX enzyme activity decreased significantly (at 0.05 level) with an increase of Zn concentrations. Under SNP concentration, the maximum activity of enzyme was found in Zn+SNP100 and the minimum activity was identified in Zn+SNP200 (*Fig. 5*). Root APX enzyme activity under Zn stress increased significantly (0.05 level) from Zn0 to Zn100 and decreased significantly (0.05 level) with elevated concentrations of Zn. In addition, under different concentrations of Zn treatments and SNP, showed that enzyme activity significantly decreased (0.05 level) in Zn+SNP200 and significantly increased (0.05 level) in Zn+SNP100 (*Fig. 6*).

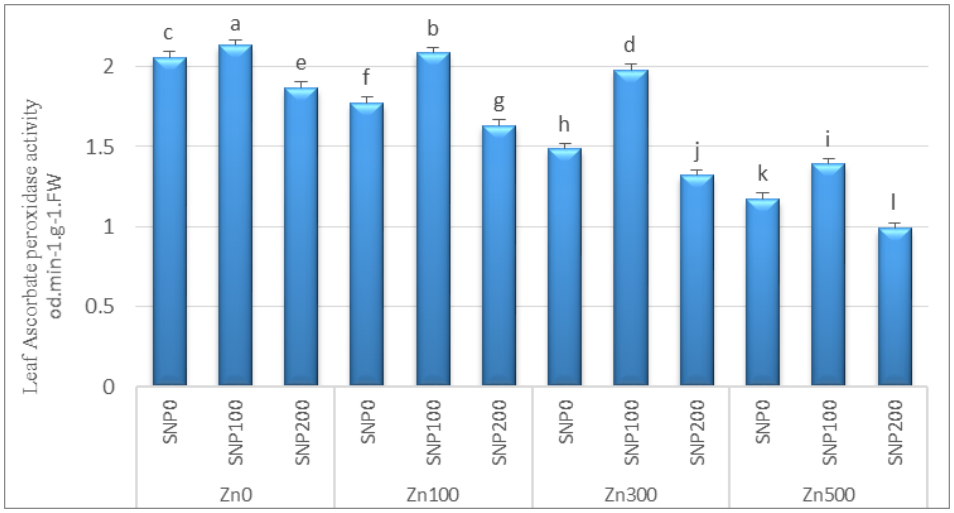

Figure 5. *Effects of different concentrations of SNP on ascorbate peroxidase activity in leaf of Plantago major under Zn stress*

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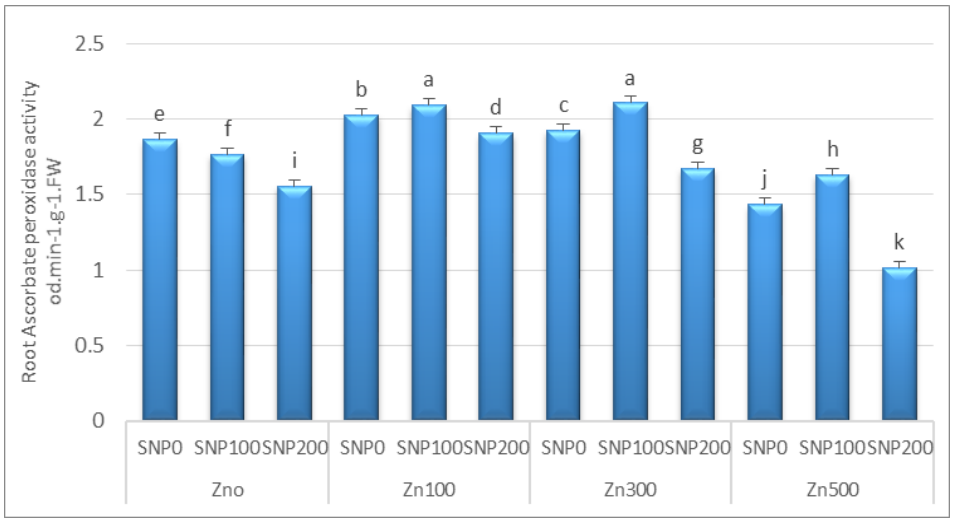

Figure 6. *Effects of different concentrations of SNP on ascorbate peroxidase activity in root of Plantago major under Zn stress*

Superoxide dismutase enzyme activity

In leaf and root of *Plantago major*, the highest level of SOD activity was seen at 100 µM concentrations of Zn. Under SNP concentration, enzyme activity decreased significantly (0.05 level) by increasing SNP. Application of SNP to Zn concentrations shows that the highest level of activity was seen at Zn (0,100,300,500) +SNP100 and the lowest level of activity was seen at Zn (0,100,300,500) +SNP200. Hence, SOD activity was decreased depending on Zn concentrations, but the application of SNP200 µM caused a significant increase in SOD activity (*Figs. 7* and *8*).

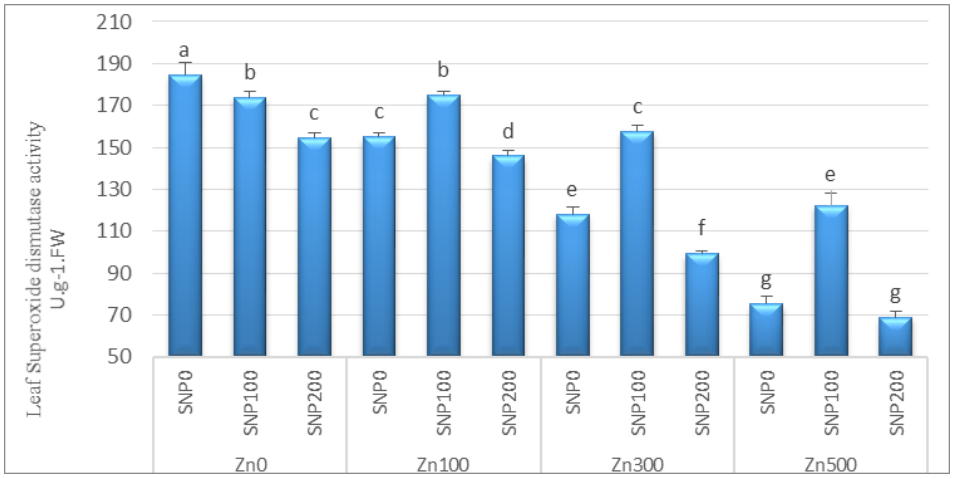

Figure 7. *Effects of different concentrations of SNP on superoxide dismutase activity in leaf of Plantago major under Zn stress*

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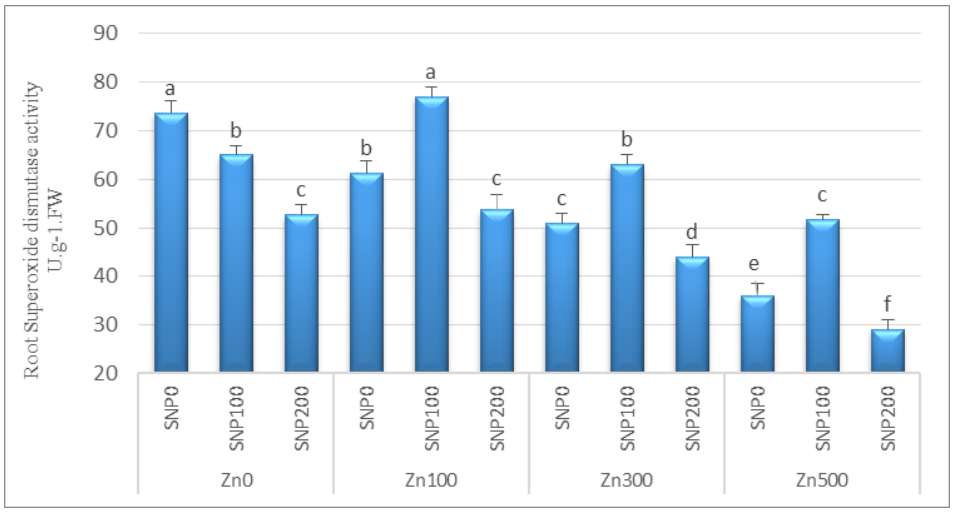

Figure 8. *Effects of different concentrations of SNP on superoxide dismutase activity in root of Plantago major under Zn stress*

Discussion

In our research, different NO concentrations were applied in Zn-treated growth medium. We investigated the physiological specifications of *Plantago* under Zn stress by using different NO concentration, aiming at finding a suitable concentration of NO to alleviate Zn toxicity effectively. The results of the present study indicated that application of Zn (100, 300 and, 500 μM) especially concentration of 500 μM decreased the dry weight of *Plantago*. However, simultaneous application of low concentration of NO (100 μM) increased the dry weight. Zn-induced growth inhibition was reflected by total dry weight. Inhibition of growth in *Plantago* plants might be the result of Zn caused variation of metabolic processes, such as oxidative damage, nutrient uptake and photosynthesis. Sheldon and Menzies (2005) reported similar results on Rhodes grass. However, Zn-induced inhibition was significantly reduced by the lower concentration of No. The mitigation effect of lower concentration of NO might be that NO induced the improvement of in photosynthesis by increasing chlorophyll content, counteracted oxidative damage by decreasing the generation of ROS. When the higher NO concentration was applied in *Plantago* plants, the mitigation effect was not obvious.

In this study, chlorophyll content showed maximum at 100 μM of Zn and it was decreased beyond that concentration. The decrease in chlorophyll content is believed to be because of: (1) inhibition of enzymes associated with chlorophyll biosynthesis (John et al., 2009); (2) inhibition of uptake and transportation of other metal elements such as Mn and Fe by adversary effects (Jayakumar et al., 2009; John et al., 2009). These results are in agreement with Yuanjie et al. (2013) who reported that Cu significantly decrease total chlorophyll, chl. a and b as compared with the control. The applications of different concentrations of SNP changed the chlorophyll contents in ryegrass. In another research, a similar reduction in total chlorophyll content under heavy metal toxicity was observed in *Atriplex halimus* (Brahim

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and Mohamed, 2011) and tobacco plants (Kheiry et al., 2016). Similar decrease in chlorophyll content under Cu stress was reported in *Atriplex halimus*. Our results shows that the application of exogenous NO at lower concentration (100 μM) alleviated Zn-induced decrease in chlorophyll content. Our results indicated that NO mediated improvement of chlorophyll contents played a role in the enhancement of photosynthesis.

As an active redox metal, Zn is able to induce the overproduction of ROS, such as hydrogen peroxide (H_2O_2), which in turn lead to lipid peroxidation and oxidative stress (Zhang et al., 2009). Results of this study showed that high concentrations of Zn, especially in 500 μM induced over accumulation of H_2O_2 and MDA in leaves and roots, leading to the oxidative damage in *Plantago* plants. However, application low concentration of NO (100 μM) alleviated H_2O_2 under Zn stress, protected cell membrane from peroxiding and decreased the accumulation of MDA. Similar to our results an increase in MDA and H_2O_2 content under excess nickel has been reported in *Eleusin*e *coracana* L. (Viswanath et al., 2016).

The levels of lipid peroxidation in the plant cells are measured by the determination of their MDA content, a breakdown product of lipid peroxidation. A high level of MDA is expressive of an enhanced formation of ROS and oxidative damage. In fact, ROS delete hydrogen from unsaturated fatty acids and generates lipid radicals and reactive aldehydes, which distort the lipid bilayer (Mishra et al., 2006). The results in the present work are in adaptation with the explanation of Panda et al. (2003), who reported heavy metal induced oxidative stress in Wheat leaves cell. Zhang et al. (2014) investigated that excess Zn altered the redox status of the aquatic plants (*Hydrilla verticillata*). In support, Chao et al. (2008) reported rapid generation of H_2O_2 by Zn-treated plant cells.

To cope with ROS and alleviate their toxic effects, plants have different antioxidation mechanisms (mediated by both enzymatic and non enzymatic antioxidants) operational in them to take care of the deleterious ROS (Puthur, 2016). Antioxidant system plays an important role in plant tolerance to stress conditions, which is based on the fact that the activity of one or more of these enzymes or antioxidant substances in general increase in plants when exposed to stressful condition and these enhances are related to increased stress tolerance (Fecht-Christoffers et al., 2003).

In our experiment, results shows that activities of antioxidant enzymes such as SOD and APX were decreased with increasing Zn concentrations but POD and CAT were increased. However, supply of exogenous NO, mainly 100 μM improved the Zn-alteration on antioxidant enzyme activity, which played a role in alleviating Zn-induced oxidative damage. Many studies have found a decrease (Malar et al., 2014) or increase (Xu et al., 2014) in activity of antioxidant enzymes under metal stress.

Previous reports revealed a variable response of an increase or decrease in SOD activity in plants exposed to different metals including Zn (Dixit et al., 2001). SOD is an essential component of antioxidant defense system in plants and it dismutase two superoxide radicals to water and molecular oxygen (Magdy and Azooz, 2013). A reduction in SOD activity in the metal treated plants has been attributed to an inactivation of the enzyme by H_2O_2 that is formed in different cellular compartments where SOD catalyzes the scavenging of superoxide radicals. SOD activity has been reported to increase under Zn toxicity (Kupper et al., 2007). Increase in SOD activity in response to stress appears to be probably due to de-novo synthesis of the enzymes protein (Erdei et al., 2002). Magdy and Azooz (2013) indicated that Zn treatment induced the activities of CAT and APX in shoots of *Hibiscus*

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esculentus. CAT is universally present oxidoreductase that decomposes H_20_2 to water and molecular oxygen and it is one of the key enzymes involved in removal of toxic peroxides. CAT along with APX and SOD are considered as vital enzymes within the antioxidant defense mechanism, which directly determine the cellular concentration of H_2O_2 (El-Shora, 2004). All the antioxidant enzymes studied in this work explain maximum activity in leaf compared to roots. It might be due to translocation of Zn in aerial parts as a micronutrient and this augmented the concentration of antioxidant enzymes in leaf compared to roots. The increased antioxidant enzyme activity might be ascribed to the role of NO in stimulating the expression of their genes (Xiong et al., 2010; Shi et al., 2016). Two mechanisms, which may explain NO protective action against oxidative damage, have been reported. Firstly, NO might detoxify ROS directly, such as superoxide anion, to form peroxyntrite, which is less toxic and thus limit cellular damage (Martinez et al., 2000). Secondly, NO could function as a signaling molecule, which activates the cellular antioxidant system (Lamattina et al., 2003). In the present study, the application of high NO concentration did not reduce Zn-induced ROS damage, even produced more toxic effects in *Plantago major*. However, the effects of NO depend on its concentration. Lamattina et al. (2003) reported that NO might regulate the expression of antioxidative genes to stimulate the relative enzyme activities. NO-mediated increase in one or more of these antioxidant enzymes contributed to the enhancement of antioxidative ability. Such results suggested that the protective effects of NO on oxidative damage were partly related to its role in up-regulating antioxidative ability. Universally, NO, as a recognized regulator of protein activation by S-nitrosylation, might inactivate heavy metal by modification of phytochelatins or some other ligands containing SH-group (Fecht-Christoffers et al., 2003). Finally NO can function as a signaling molecule for the cascade of events that lead to changes in gene expression under risk element stress (Procházková et al., 2012).

In conclusion ,our results demonstrated that in the hydroponics experiment, the alleviation effects of NO on Zn stress in *Plantago* needed a suitable concentration and the lower concentration of NO (100 μM) had a higher protective effect on Zn toxicity, while high concentration of NO (200 μM) did not alleviate Zn toxicity effectively. Our study proved that exogenous NO at low concentrations increased Zn tolerance in *Plantago* grown in Zn–mediated nutrient solution by (1) improving antioxidant enzyme activities and protecting against Zn–induced oxidative stress, (2) reversed the Zn toxicity effect and decreased the MDA content and H_2O_2 accumulation. This indicates that NO acts as an efficient ROS scavenger and membrane stabilizer in *Plantago* plants exposed to Zn stress.

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