

PROTECTIVE EFFECTS OF EXOGENOUS NITRIC OXIDE AGAINST LEAD TOXICITY IN LEMON BALM (*Melissa officinalis* L.)

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Abstract. This research investigated the effects of exogenous sodium nitroprusside (SNP) supplementation as nitric oxide (NO) on alleviating Pb-induced oxidative damage in lemon balm (*Melissa officinalis* L.) plants. Pb (100, 300 and 500 μ M) alone and in combination with SNP (100 and 200 μ M) were given to hydroponically grown *Melissa officinalis* L. plants. The findings suggested that in Pb-treated plants, height, total dry weight, and chlorophyll content of leaves markedly decreased while application of 100 μ M SNP alleviated the inhibitory effect of Pb on plant growth and chlorophyll content. Pb exposure caused oxidative stress by elevating lipid hydroperoxide contents of the seedlings. Application of 100 μ M SNP counteracted Pb toxicity by reducing lipid hydroperoxide contents of Pb-treated seedlings. Furthermore, it was found that the activities of antioxidant enzymes such as polyphenol oxidase, catalase, guaiacol peroxidase, and ascorbate peroxidase were increased in Pb-treated plants. The use of NO especially at low concentrations reversed Pb-induced negative effects whereas high concentrations of NO had no obvious alleviating effect on Pb toxicity in *Melissa officinalis* L. On the other hand, application of 100 μ M SNP could function as a defense mechanism of the plant against Pb toxicity and mitigate Pb stress.

Keywords: medicinal plant, oxidative stress, lead pollution, antioxidant response, sodium nitroprusside

Introduction

Heavy metal pollution has become a prominent environmental problem around the world. Among heavy metals, lead (Pb) is one of the most dangerous pollutants of the environment and Pb pollution in the air, water, and agricultural soil is an ecological concern due to its impact on human health and the environment. Although it is not an essential nutrient for plants, a great portion of Pb is easily taken up by plants from the soil and accumulated in roots while only a small fraction is translocated upward to the aerial parts of plant (Bai et al., 2015). Pb exposure affects growth and physiological parameters and leads to decreases in germination percent, length, and dry mass of roots and shoots, disturbing mineral nutrition and reducing cell division (Singh et al., 2011). In most studies, Pb has been known to induce oxidative stress through overproduction of reactive oxygen species (ROS) and hydrogen peroxide (H₂O₂), which in turn act on the unsaturated lipids in the cell membranes, ultimately leading to lipid peroxidation and damages in cell membranes (Kumar et al., 2013).

Plants are exposed to a wide range of pollutants, particularly heavy metals. However, they are different in their responses to heavy metal pollution. For instance, while some are sensitive to Pb, others show tolerance for this heavy metal and are able to take in considerable amounts of Pb (Nemati et al., 2013). In fact, plants have adopted

specific ways to deal with pollution. Blocking the heavy metals' entrance into the cell via exclusion or binding them to cell wall is the first major mechanism of detoxification and this is an important mechanism for Pb pollution (Antosiewicz and Wierzbicka, 1999). Also, some plants have improved an anti-oxidative system, including anti-oxidative enzymes such as guaiacol peroxidase (GPOX), catalase (CAT), and ascorbate peroxidase (APX) (Cakmak and Horst, 1991).

Nitric oxide (NO), a free radical in living organisms, is considered a key signaling molecule and a phytohormone which has important roles in various physiological processes of plants such as germination, growth, senescence, photosynthesis, and response mechanisms to specific environmental stresses (Del Rio et al., 2004). It is reported to protect the plants against toxicity of reactive oxygen species (ROS), enhance their tolerance to abiotic stress, and improve their defense response (Besson-Bard et al., 2008; Neill et al., 2008). The application of a NO donor, SNP, confers tolerance to various abiotic stresses in plants by enhancing their antioxidant defense system under stress conditions (Xu et al., 2010). Nitroprusside plays its role through the production of compounds involved in detoxification of H₂O₂ and antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (AXP), and guaiacol peroxidase (GPOX) (Tewari et al., 2006). NO has been found to reduce Pb uptake in *Arabidopsis thaliana*, thereby reducing toxicity symptoms (Phang et al., 2011). It also influences gene expression in response to oxidative stress in *Zea mays* leaves (Hermes et al., 2011). Nitroprusside decreased absorption of cadmium in *Melia azedarach* cuttings and improved some growth parameters under cadmium stress treatment (Arany et al., 2015). It was recently reported that exogenous application of SNP (as a NO donor) alleviated the hostile effects of abiotic stresses induced by heavy metals such as As (Hasanuzzaman and Fujita, 2013), Br and Al (Aftab et al., 2012) and Cu (Zhang et al., 2009). Pretreatment of cowpea seeds with SNP as NO donor before exposure to Pb had a protective effect against Pb toxicity conducting to an improvement of the chlorophyll value, RWC, and net photosynthetic rate by increasing antioxidant enzyme activities (Sadeghipour, 2015). *Melissa officinalis* L contains some phenolic and flavonoid compounds such as rosmarinic acid. Phenolic contents in plants have some antioxidant properties (Chen et al., 2001). This plant is widely used around the world because of its medicinal properties. In the *Melissa officinalis* L. plants treated with sodium nitroprusside, increase in concentrations of sodium nitroprusside led to an increase in oxidative stress molecules and malondialdehyde (Esmailzadeh et al., 2015).

Lemon balm (*Melissa officinalis* L.) is a member of the Lamiaceae family, which spreads widely from the western part of Europe (Ulbricht et al., 2005) to western and central parts of Iran. This plant is widely used around the world because of its medicinal properties. However, there is a paucity of data regarding the role of NO in alleviating Pb-induced toxicity. With that background in mind, we hypothesized that NO may ameliorate Pb-induced toxic effects in *Melissa officinalis* L. The present work aimed at investigating the role of exogenously supplied SNP (NO donor) in alleviating Pb stress in *Melissa officinalis* L.

Materials and Methods

Plant material and culture conditions

Melissa Officinalis L. seeds were cultured in perlite puts 2 centimeters deep at equal distances before they were sterilized with 5% sodium hypochlorite for 15 min and

washed thoroughly with distilled water. The pots were irrigated by distilled water for 10 days. After germination, the seeds were nourished with Hoagland feeding solution for 15 days. Irrigation was continued during 3-leave stage with Hoagland solutions containing different concentrations of Pb (NO₃)₂ (0,100, 300, and 500 μM) and sodium nitroprusside (0,100, and 200 μM) alone and in combination for two weeks. Our experiment was performed under regulated conditions, daily temperature of 25/17° C and 60 ± 5% relative humidity. For the assessment of plant dry matter content, the plants were dried at 80° C for 48 h, to produce a constant weight. This research was conducted under the standard conditions at Sari Agricultural Research Center in Iran.

Determination of Photosynthetic pigments

The leaves were chopped into small pieces that were extracted with 80% acetone. The absorbance was measured at 645 nm and 663 nm for chlorophyll a and b, respectively. Then photosynthetic pigments (chlorophyll a and b) were assayed as per the method of Litehtenthaler and Wellburn (1983) formulae:

$$\text{Chl a (mg g}^{-1} \text{ leaf fresh weight)} = [12.7 (\text{OD}663) - 2.69 (\text{OD}645)] \times V/1000 \times W$$

$$\text{Chl b (mg g}^{-1} \text{ leaf fresh weight)} = [22.9 (\text{OD}645) - 4.68 (\text{OD} 663)] \times V/1000 \times W$$

$$\text{Total Chl (mg g}^{-1} \text{ leaf fresh weight)} = [20.2 (\text{OD}645) - 8.02 (\text{OD} 663)] \times V/1000 \times W$$

where:

OD= Optical Density

V = Volume of Sample

W = Weight of Sample

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 g) were 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 nM g⁻¹ FW.

Determination of antioxidant enzymes

For extraction of antioxidative enzymes, shoots and roots were homogenized with 50 mM Na₂HPO₄-NaH₂PO₄ buffer (pH 7.8) including 0.2 mM ethylene diamine tetra acetic acid (EDTA) and 2% insoluble polyvinyl pyrrolidone in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determining enzyme activities. The whole extraction procedure was carried out at 4 °C.

PPO activity was determined by measuring the increase in the absorbance at 420 nm for catechol and 4-methylcatechol substrates and at 320 nm for pyrogallol substrate (Dogan et al., 2007). CAT activity was measured as the decrease in the absorbance at 240 nm due to the decline of extinguishment of H₂O₂ by the procedure of Patra et al. (1978). APX activity was measured by the decline in the absorbance at 290 nm, as ascorbate was oxidized (Nakano and Asada, 1981). GPX activity was calculated by using the extinction coefficient of 26.6 M⁻¹ cm⁻¹ for H₂O₂ at 436 nm and was expressed as nKat/mg⁻¹ of protein. The 10 % gel was stained by the procedure of Hamill and Brewbaker (1969).

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

Growth, malondialdehyde content and photosynthetic pigments

Total dry weight of Pb-treated plant was reduced significantly compared with control (Table 1). Different concentrations of Pb (0, 100, 300 and 500 µM) and interaction with sodium nitroprusside (100 and 200 µM) on plant growth, expressed that dry weight was increased in SNP100, however, 200 µM SNP into Pb-treated solution was not diminished and dry weight of plant was decline significantly.

Effects of different concentrations of Pb (0, 100, 300 and 500 µM) and their interaction with sodium nitroprusside (100 and 200 µM) on plant growth expressed as height are shown in Table 1. Pb exposure inhibited the growth of *Melissa officinalis* significantly compared with control (Pb 0 Snp 0); however, this inhibition was moderated by the additions of 100 µM SNP. On the other hand, after application of 200 µM SNP into Pb-treated solution, Pb-induced inhibition on plant growth was not diminished and height of Pb-treated plant was reduced significantly (Table 1).

According to Table 1, the rate of MDA in shoots and roots increased in Pb-treated plants in comparison with the control group, significantly (P<0.05). Increasing MDA content resulted in increased lipid peroxidation in the metal-exposed plants. Under Pb stress, application of low SNP concentrations (SNP 100 µM) decreased MDA content, but increasing SNP concentrations (SNP200 µM) to Pb treatments did not alleviate the effects of Pb stress on lipid peroxidation (Table 1).

The rise in shoot chlorophyll a and b contents of *Melissa officinalis* plants exposed to Pb stress was statistically significant compared with control group; however, application of 100 µM SNP alleviated Pb toxicity in the photosynthetic system. High concentration of SNP (200 µM) had no mitigating effects on decreasing chlorophyll contents and both chlorophyll a and b contents were reduced, meaning fully compared to others (Table 1).

Table 1. Effects of different concentrations of SNP (0, 100 and 200 μM) on total dry weight, Chl. a and b, leaf and root MDA content, leaf and root H₂O₂ content in *Melissa officinalis* under Pb stress (0, 100, 300, and 500 μM).

Pb	SNP	Height (cm)	Total dry weight (g)	Chl a (mg/g FW)	Chl b (mg/g FW)	Leaf MDA content (nmol.g ⁻¹ .FW)	Root MDA content (nmol.g ⁻¹ .FW)
0	0	22.85 a	4.6 b	2.803 ab	0.8 a	26.84 h	17.85 h
	100	22.25 ab	4.843 a	2.853 a	0.801 a	27.4 gh	17.96 h
	200	18.82 d	4.08 cd	2.61 bcd	0.767 b	33.77 de	21.44 g
100	0	21.58 bc	4.236 c	2.463 de	0.767 b	35.67 cd	25.43 f
	100	23.19 a	4.826 ab	2.706 abc	0.812 a	29.9 fg	21.61 g
	200	18.96 d	4.13 cd	2.346 e	0.757 b	37.72 c	26.94 ef
300	0	20.64 c	3.903 de	2.053 f	0.66 d	37.73 c	32.64 d
	100	22.29 ab	4.116 cd	2.496 cde	0.727 c	32.41 ef	28.3 e
	200	18.02 de	3.676 e	1.786 g	0.634 e	38.51 c	34.68 c
500	0	16.67 f	3.406 f	1.473 h	0.502 g	41.85 b	37.78 b
	100	17.79 e	3.686 e	2.056 f	0.581 f	37.5 c	32.98 cd
	200	16.1 f	3.34 f	1.266 h	0.453 h	46.81 a	41.41 a

Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

Enzymes activity

Under Pb stress condition, CAT enzyme activity in shoots and roots of *Melissa officinalis* increased significantly ($P \leq 0.05$). In SNP-treated plant, both concentrations of nitroprusside increased catalase activity significantly ($P \leq 0.05$) and the increase was more pronounced at SNP100 concentration. Interaction between Pb and SNP ed that CAT enzyme activity increased significantly in Pb+SNP100 μM but there was a significant decrease at Pb+SNP200 μM . It seems that low concentration of NO enhances negative effects of Pb stress (Figs. 1 and 2).

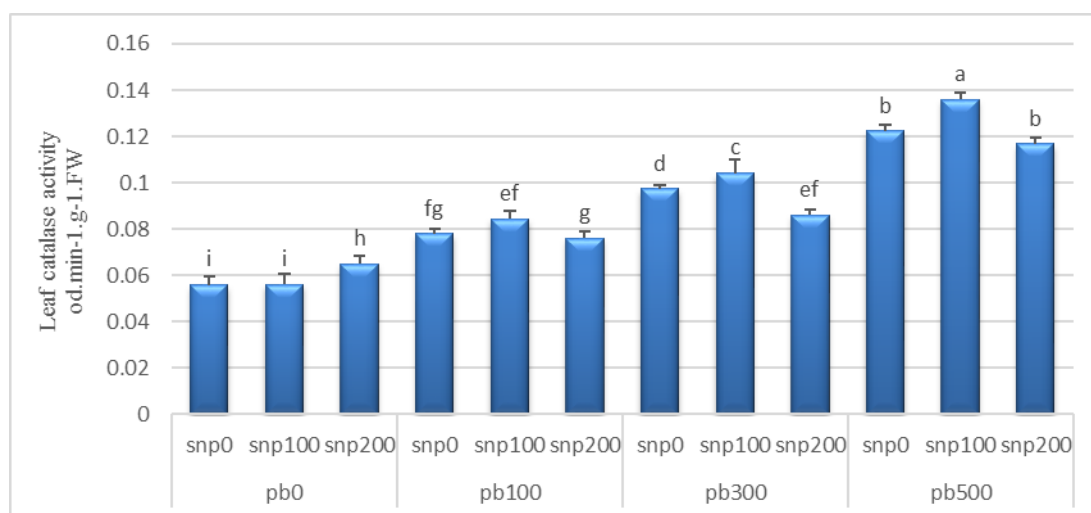


Figure 1. Effects of different concentrations of SNP on catalase activity in shoots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

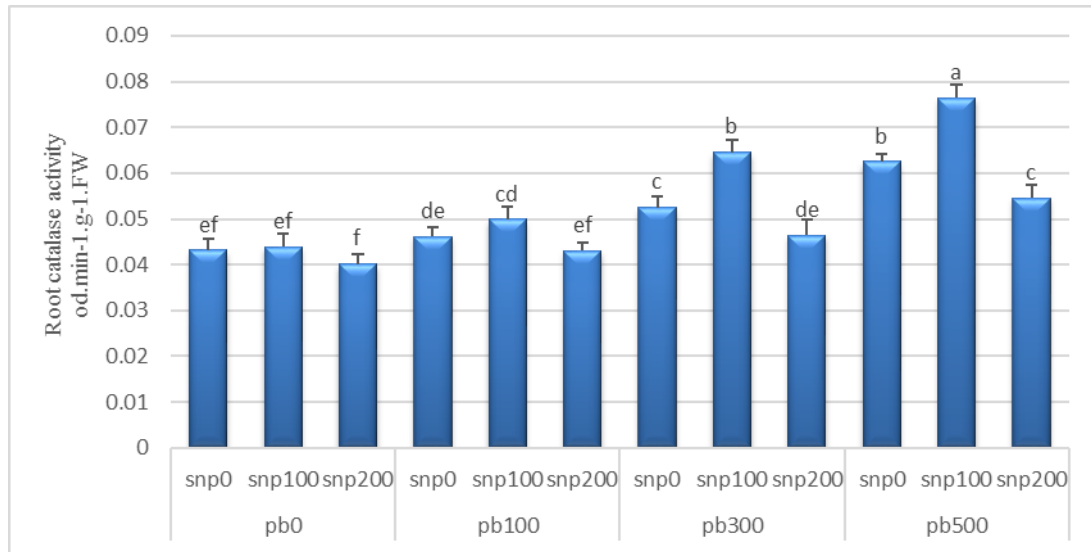


Figure 2. Effects of different concentrations of SNP on catalase activity in roots of *Melissa officinalis* under Pb stress ($P \leq 0.05$). Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

With an increase in Pb concentrations, leaf APX enzyme activity decreased significantly ($P \leq 0.05$). Also, with increase in SNP concentrations, APX enzyme activity decreased. Under different concentrations of Pb and SNP, the maximum and minimum APX enzyme activities were recorded in Pb+SNP100 and Pb+SNP200, respectively (Fig. 3).

In roots, under Pb stress APX enzyme activity increased significantly ($P \leq 0.05$). In addition, in both roots and leaves, with increase in SNP concentrations, APX enzyme activity decreased. Also, APX enzyme activity significantly decreased ($P \leq 0.05$) in Pb+SNP200 treatment while there was a significant increase in the activity of this enzyme in the treatment containing Pb+SNP100 (Fig. 4).

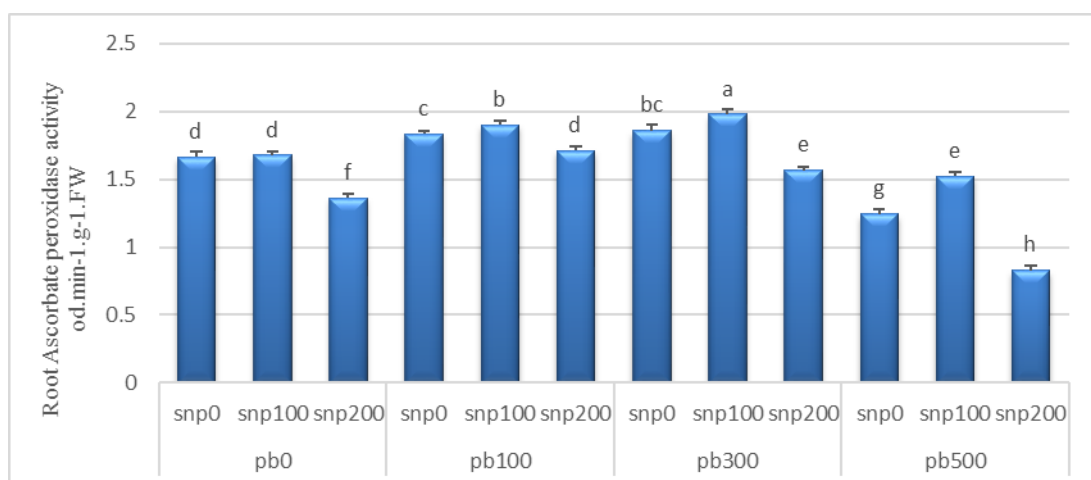


Figure 3. Effects of different concentrations of SNP on ascorbate peroxidase in roots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

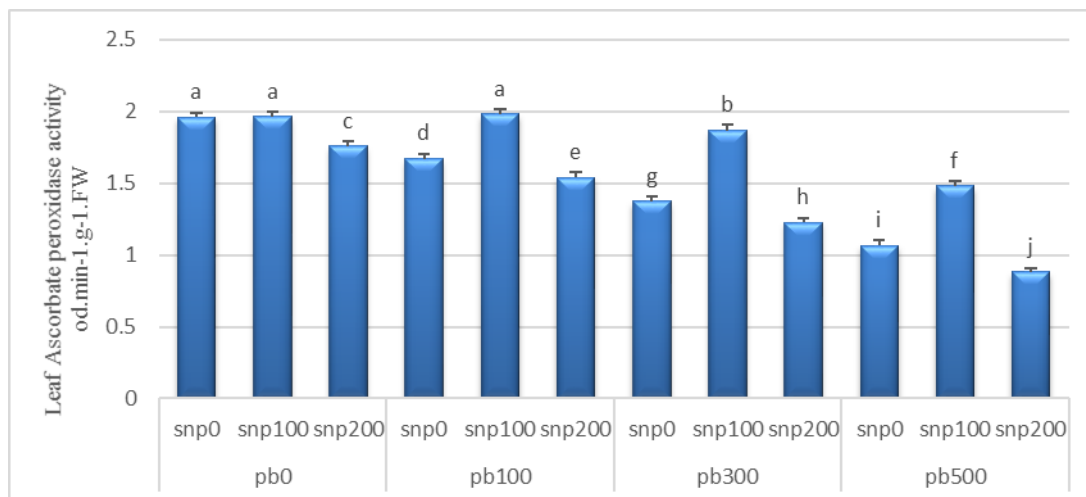


Figure 4. Effects of different concentrations of SNP on ascorbate peroxidase in shoots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

In leaves of *Melissa officinalis* under Pb stress, guaiacol enzyme activity did not change with an increase in lead concentration but under SNP treatment, enzyme activity decreased significantly ($P < 0.05$) with increasing SNP concentrations and in fact, SNP 100 μM showed the greatest increase in enzyme activity. The highest level of guaiacol activity was seen in the treatment containing Pb 300 μM + SNP100 μM .

In root, under SNP concentration, enzyme activity was unaltered with increasing SNP. Also, with increasing concentrations of lead, enzyme activity increased in roots and the increase was particularly remarkable at Pb 300 μM . By application of SNP to Pb-treated plants, the highest level of guaiacol enzyme activity was seen at Pb (100 μM and 300 μM) + SNP100 μM while the lowest activity was seen at Pb 500 μM + SNP 200 μM (Figs. 5 and 6).

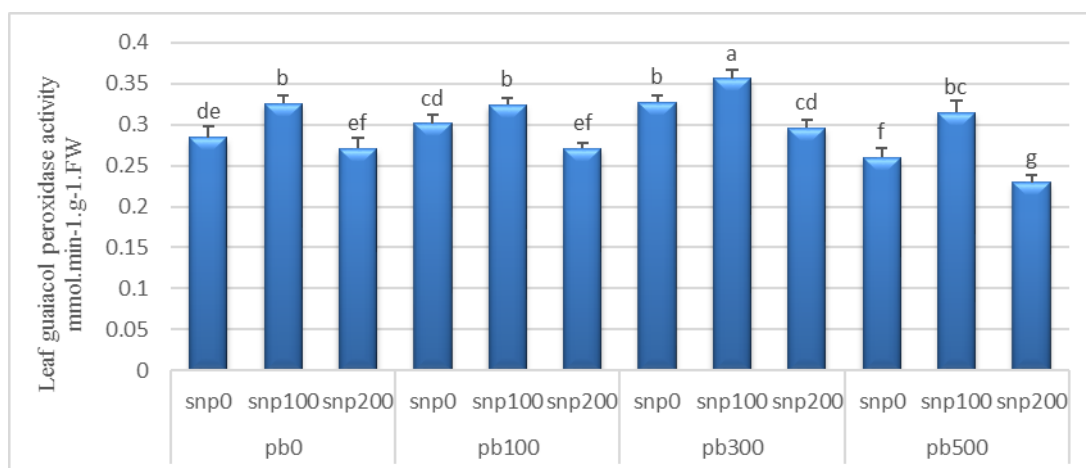


Figure 5. Effects of different concentrations of SNP on guaiacol peroxidase activity in shoot of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

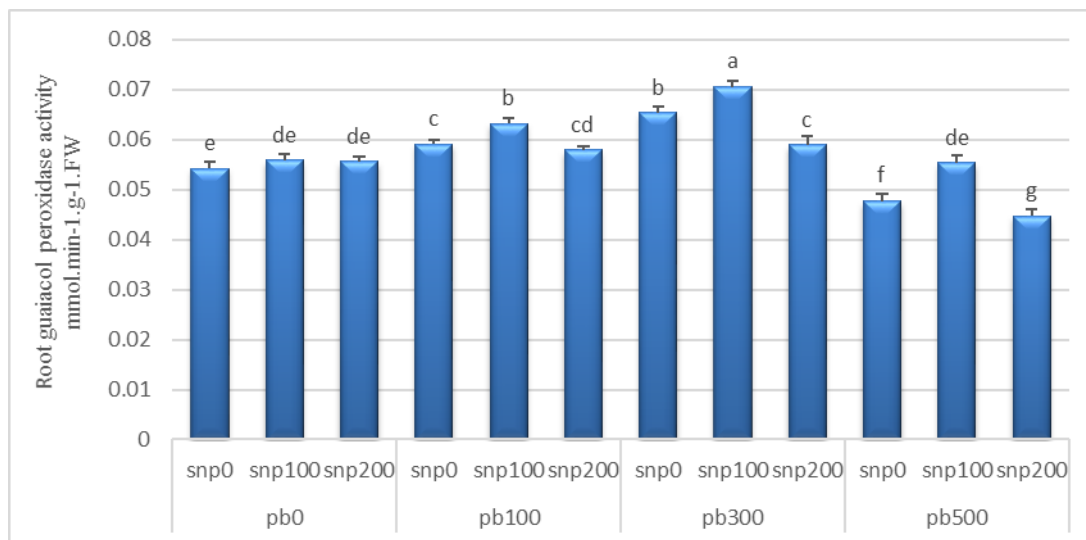


Figure 6. Effects of different concentrations of SNP on guaiacol peroxidase activity in roots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

In leaves and roots of *Melissa officinalis* under Pb stress, polyphenol oxidase activity increase with increasing lead concentration except in Pb 500 μM . Under SNP concentrations, enzyme activity increased significantly ($P \leq 0.05$) with increasing SNP and SNP 100 μM showed the greatest increase in polyphenol oxidase activity. The highest level of polyphenol oxidase activity was seen at Pb (100 μM and 300 μM) + SNP 100 μM . Under SNP treatment, the highest level of polyphenol oxidase activity was seen at Pb (100 μM and 300 μM) + SNP 100 μM and the lowest level of activity was seen at Pb 500 μM + SNP 200 μM (Figs. 7 and 8).

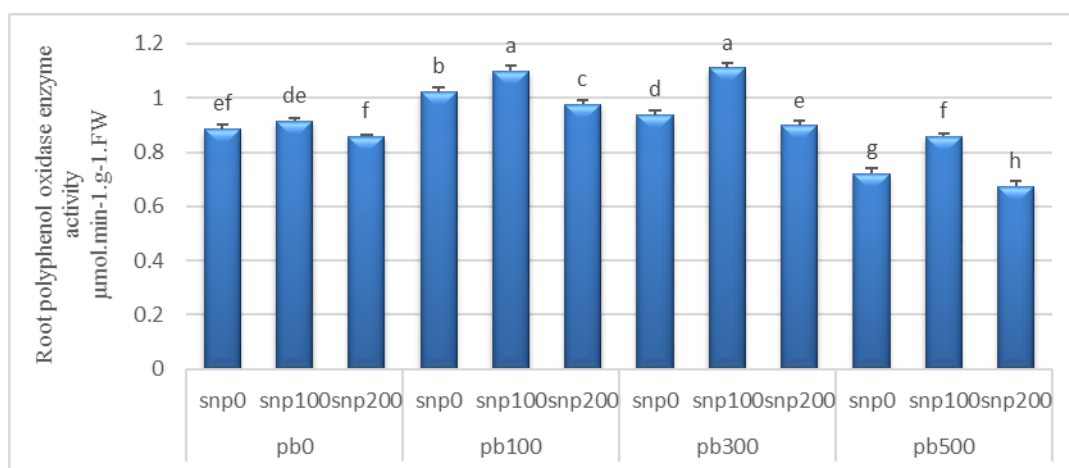


Figure 7. Effects of different concentrations of SNP on polyphenol oxidase activity in roots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

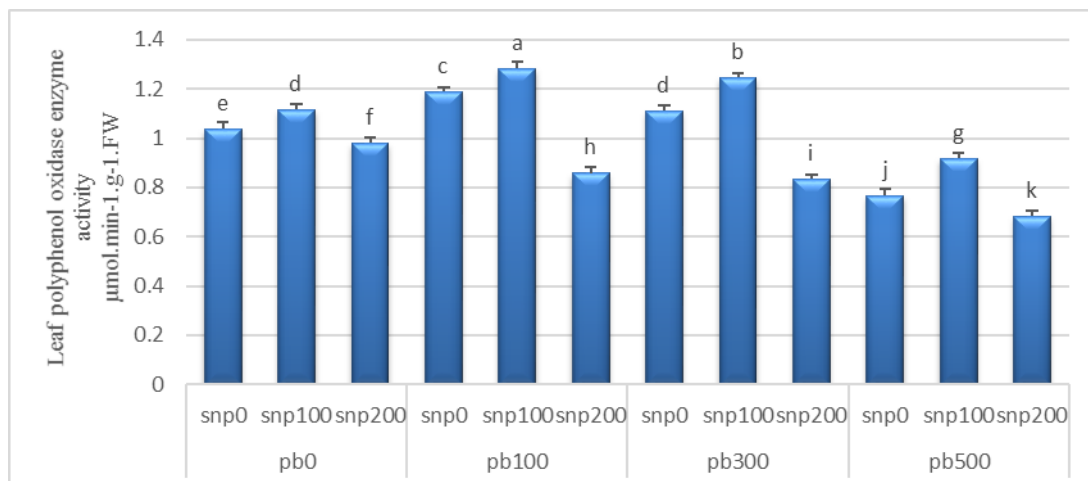


Figure 8. Effects of different concentrations of SNP on polyphenol oxidase activity in shoots of *Melissa officinalis* under Pb stress. Means followed by the same letter are not significantly different ($P < 0.05$) according to LSD test ($n=3$).

Discussion

Pb is a toxic heavy metal that exerts adverse effects on morphology and growth of plants causing inhibition of enzyme activities. It has been suggested that exogenous application of the NO donor, sodium nitroprusside (SNP), enhances plant tolerance to heavy metals (Kumari et al., 2010) and oxidative stress (Esim and Atici, 2013). In the present study, the probable effects of exogenous NO was investigated on reducing lead toxicity in *Melissa officinalis*. Different concentrations of NO were applied in the Pb-treated plants and the physiological parameters of *Melissa officinalis* under Pb Stress were investigated under different NO concentrations.

Plant biomass is a good indicator for characterizing the growth performance of plants in the presence of heavy metals. Results indicated that application of Pb (100, 300, and 500 µM) especially concentration of 500 µM decreased the dry weight of *Melissa officinalis*. However, simultaneous application of low concentration of NO (100 µM) increased the dry weight and height of *Melissa officinalis*. Pb-induced growth inhibition in this study was reflected by total dry weight. Similar response to lead treatment was previously reported in various plants (Brunet et al., 2009). Decrease in growth parameters (dry weight and height) in *Melissa officinalis* plants might be the result of changes in metabolic processes, e.g. oxidative damage, nutrient uptake, and photosynthesis or it might be associated with the inhibition of mitotic index observed under Pb heavy metal treatment (Sheldon and Menzies, 2005). Previous studies on the mechanism of Pb toxicity suggested that Pb binds to nucleic acids and causes aggregation and condensation of chromatin, inhibiting the process of replication and transcription and ultimately affecting cell division and plant growth (Johnson, 1998). In our study, Pb-induced inhibition was significantly reduced by the lower concentration of NO. The mitigation effect of lower concentration of NO might be because NO improved photosynthesis by increasing chlorophyll content and counteracted oxidative damage by decreasing the generation of ROS. When the higher NO concentration was applied in *Melissa officinalis* plants, the mitigation effect was not obvious.

In the present study, the chlorophyll content significantly decreased with the increasing concentrations of externally supplied Pb in *Melissa officinalis*. This reduction

in chlorophyll content can be regarded as a specific response of the plants to metal stress, which resulted in chlorophyll degradation and inhibition of photosynthesis (Gajewska et al., 2006). Chlorophyll content showed maximum range at Pb (100 μ M) and it was decreased beyond that concentration. Heavy metals inhibit chlorophyll and other pigments biosynthesis. Lead may impair the uptake of essential elements, such as Mg and Fe, for chlorophyll biosynthesis, substitute divalent cations, prevent enzyme activity like aminolevulinic acid dehydratase and increase chlorophyll biodegradation by enhancing chlorophyllase activity (Nareshkumar et al., 2014; John et al., 2009; Prasad and Prasad, 1987). Therefore, reducing the amount of chlorophyll can be due to several factors, namely, inhibition of enzymes associated with chlorophyll biosynthesis (John et al., 2009), inhibition of Calvin cycle enzyme activity (Sharma and Dubey, 2005), obstruction of electron transportation (Pourrut et al., 2011), and stomatal closure and distortion of chloroplast ultrastructure (Bharwana et al., 2014; Bai et al., 2015). Also, it may be the result of interaction of Pb with SH group of enzymes of chlorophyll biosynthesis as well as lipid peroxidation-mediated degradation as indicated by Singh et al. (2006). Similar cause was suggested by Tanyolac et al. (2007) in maize (*Zea mays* L.) under Cu stress.

Similarly, reduction in the chlorophyll content has been reported in many plant species exposed to Pb (Bharwana et al., 2014; Malar et al., 2014). Our results indicated that NO-mediated improvement of chlorophyll contents played a role in the enhancement of photosynthesis. In this experiment, exogenous NO at lower concentration (100 μ M) increased the chlorophyll content under Pb toxicity but high concentration (200 μ M) of NO had no positive effect and chlorophyll content significantly decreased at Pb (500 μ M) ($P < 0.05$). A high NO-induced accumulation of chlorophyll was also observed under Cd stress in Rapeseed and tomato (Jhanji et al., 2012) and wheat under As-induced oxidative stress (Hasanuzzaman and Fujita, 2013).

It was found that NO raised the antioxidant enzyme activity and consequently reduced chlorophyll desolation induced by ROS. On the other hand, NO also protected chlorophyll by increasing the uptake of Fe and Mg under Pb stress (Bai et al., 2015). In this way, NO protects chlorophyll by improving its biosynthesis and reducing its destruction under Pb stress conditions.

In our study, the MDA level was significantly increased by Pb treatment. Increase in the concentration of lead was observed to increase the level of malondialdehyde production. MDA is the final product of peroxidation of membrane lipids and accumulates when the plants are subjected to oxidative stress. Therefore, MDA level is routinely used as an index of lipid peroxidation under stress conditions. A high level of MDA is demonstrative of an increased formation of ROS and oxidative damage. Actually, ROS reject hydrogen from unsaturated fatty acids and produce lipid radicals and reactive aldehydes, which deform the lipid bilayer (Kumar et al., 2013). Pb is known to induce oxidative stress in plants due to the elevated manufacture of ROS (Singh et al., 2011). In this work, increasing MDA levels showed that oxidative stress and lipid peroxidation of membranes were induced by Pb stress in *Melissa officinalis* plants. This is supported by many studies, e.g., Pb toxicity caused oxidative damage and elevated MDA content in mung bean (Hassan and Mansoor, 2014), *elsholtzia argyi* (Islam et al., 2007), and cotton (Bharwana et al., 2014).

It was also observed in the present study that application of SNP 100 μ M under Pb stress conditions inverted the Pb toxicity effect and decreased the MDA content compared to Pb treatment alone. However, the use of high concentrations of NO did not

mitigate the oxidative effects of lead. NO moderated Pb stress in *Melissa officinalis* plants by averting oxidative damage via the enhancement of antioxidant enzyme activity. This suggests that NO acts as an efficient ROS scavenger and/or membrane stabilizer in *Melissa officinalis* plants subjected to Pb stress. The reaction of NO with ROS could prevent the damage to the membranes. It has been demonstrated that the reaction of NO with lipid alcoxyl (LO) and peroxy (LOO-) radicals is quick (Beligni and Lamattina, 1999). Nitric oxide has also been reported to inhibit ion drip from plant tissue and protect plants against membrane damage due to lipid peroxidation under various stress conditions. This role of NO has been observed in heavy metal stress induced by Cd (Kumari et al., 2010), Br (Esim and Atici, 2013), and Al (Wang and Yang, 2005).

Under normal conditions, ROS are produced at a low concentration and there is a balance between the generation and alleviation of ROS. This balance may be disturbed by many environmental stresses. In order to protect themselves against oxidative stress, plants have developed a compound antioxidative protection system for scavenging ROS (Sharma et al., 2012). In order to scavenge ROS and to escape oxidative injury, plants possess an antioxidative system including antioxidative enzymes. Antioxidant system plays an important role in the plants' 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 increases in plants exposed to stressful condition and this enhancement is related to increased stress tolerance (Fecht-Christoffers et al., 2003) and is considered as an efficient system for detoxification and cleaning up of the toxic oxygen species through an adaptive mechanism involving upregulation of anti-oxidative enzymes like SOD, CAT, PPO, APX, and GPX (Foyer et al., 1994).

Our results show that activities of enzymes had various effects on *Melissa officinalis* under Pb-stress. APX enzyme activity decreased with increasing Pb concentrations but CAT and PPO enzyme activities increased while GPX enzyme activity was unchanged compared to the controls. However, application of exogenous NO, particularly 100 μ M improved antioxidant enzyme activity. Modification in the activity of antioxidative enzymes with Cd stress has been reported by a number of researchers (Mishra et al., 2008; Laspina et al., 2005).

Our results show that the maximum activity of CAT was found at Pb 500 μ M and we found that with increasing concentration of the lead, activity of CAT increased. CAT is a generally present oxidoreductase that disintegrates H_2O_2 to water and molecular oxygen, and it is one of the key enzymes involved in the ejection of toxic peroxides. Increase in CAT activity can be illustrated by an increase in its substrate, to support the level of H_2O_2 as an adaptive mechanism of the plants (Reddy et al., 2005). Therefore, reduction in the activities of CAT might be due to the formation of a protein complex with metals that results in the structural integrity of proteins (Mohan et al., 1997). At higher concentration of Pb the activity of protein-based enzyme might be reduced due to the effect of ROS through reduced enzyme synthesis or change in assembly of its subunits (Verma and Dubey, 2003). CAT enzyme can play a role in controlling H_2O_2 level in cells. In subcellular compartments of pea root cells increased catalase activity was observed when plants were grown in nutrient $Pb(NO_3)_2$. However, a decline in the activity of catalase with an increased heavy metal concentration has also been observed in *Lemna gibba* (Parlak and Yilmaz, 2013) and in *Becopa monnera* (Mishra et al., 2006). Laspina et al. (2005) reported a decline in CAT activity under Cd stress. Thus, it

seems that the differences in activities of antioxidant enzymes in heavy metal treated plants are highly dependent on species and experimental model.

Our results indicate an enhancement in the activity of GPX and PPO by the low concentration of Pb, suggesting that these enzymes work as an essential defense tool to resist Pb-induced oxidative damage in plants. GPX is located in cytosol, cell wall, vacuole, and in extracellular spaces. Increased peroxidase activity in Pb-stressed plants might be possibly due to increased release of peroxidases localized in the cell walls. Under sub lethal salinity and metal toxicity conditions, level of peroxidase activity has been used as potential biomarker to evaluate the intensity of stress. Similar to our findings, the activity of guaiacol peroxidase (GPX) increased in *S. Roxburghiana* plants exposed to Pb and in *Lemna minor* (Paczkowski et al., 2007).

In the present study, APX activity was different in shoots and roots of *Melissa officinalis* under Pb stress. It was enhanced at low concentration of Pb in roots, but, decreased in shoots. APX is generally attributed to an adaptive mechanism against increase levels of ROS content produced by Pb metal ions. Enzymes of ascorbate are localized mainly in chloroplasts and in other cellular organelles and cytoplasm, where they play an important role in combating oxidative stress. The positive correlation between APX activity and excess ROS may be attributed to effective scavenging of H₂O₂ content to protect stressed plants against oxidative damage induced under lead stress. Similar to the results observed in the present study, an increase in APX activity in plants following exposure of heavy metals was reported by Malar et al. (2014) in *water hyacinth*. In *Phaseolus vulgaris* and *Pisum sativum* (Rodriguez-Serrano et al. 2006) and *Ceratophyllum demersum* (Mishra et al., 2008) APX reduced under Cd stress, but a decline in APX activity under Cd stress was reported by Gomes-Júnior et al. (2006).

Our results showed that Polyphenol Oxidase activity increased by the low concentration of Pb. Similar our study, Chinmayee et al. (2014) demonstrated that, PPO activity in *Jatropha curcas* L. increased in all plant parts under chromium, cadmium and lead stress. Also, Saffar et al. (2009) reported that in *Arabidopsis thaliana*, PPO activity might be the result of extended heavy metal stress. PPO catalyzes the formation of highly active Quinone that reacts with amino or sulfhydryl groups in proteins or enzymes. As a result, these reactions lead to changes in physical, chemical, or nutritional characteristics of proteins and, in many cases, to inactivation of enzymes including PPO (Mayer and Harel, 1979).

All the antioxidant enzymes studied in this work had maximum activity in shoots compared to roots. This might be due to moving of Pb in all parts as a micronutrient and this enhances the concentration of antioxidant enzymes in shoots compared to roots. In our study SNP (100 µM) was effective in improving all measured traits in the plants under lead stress but it had no positive effect in high concentration of Pb and this might be due to the fact that NO is itself a ROS and its dual behavior (protective or toxic) depends on the conditions (Beligni and Lamattina, 1999). The protective role is based on its capacity to regulate the level and toxicity of ROS. In many studies, the alleviation of oxidative damage by NO was attributed to the induction of activity of various ROS-scavenging enzymes (Esim and Atici, 2013). The present observation is in agreement with the previous findings on rice roots treated with As (Rodriguez-Serrano et al., 2006), and *Cassia tora* roots treated with Al (Kumari et al., 2010).

Two mechanisms have been reported which may explain NO protective action against oxidative damage. One possibility is that NO might activate antioxidant systems

to scavenge ROS. Beside the role of NO in the activation of antioxidant enzymes that scavenge ROS, it can also react with $O_2^{\bullet-}$ and generate peroxynitrite ($ONOO^-$). $ONOO^-$ is unstable and may be protonated and disintegrated to a nitrate anion and a proton, or it can react with H_2O_2 to produce a nitrite anion and oxygen (Fan et al., 2014). Second, it is now accepted that NO acts as a second messenger in plants. The cytoprotective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and to regulate the level and toxicity of ROS (Hayat et al., 2010). Present results indicate that SNP plays a protective role in APX, PPO, CAT, and GPX activity in *Melissa officinalis*. In the present study, the application of high concentration of NO did not reduce Pb-induced ROS damage. In fact it even produced more toxic effects in *Melissa officinalis*. One of the most intriguing behaviors in NO biology is its dual function as a potent oxidant and effective antioxidant. This dual role of NO might depend on its concentration as well as on the environmental conditions.

Conclusion

The present study demonstrated that Pb stress caused oxidative damage and membrane lipid peroxidation, leading to a significant decrease in chlorophyll content and shoot and root growth in *Melissa officinalis* plants. On the other hand, application of SNP as NO donor in *Melissa officinalis* under Pb stress had a protective effect against Pb toxicity by increasing antioxidant enzyme (PPO, CAT, APX, and GPX) activities leading to an improvement of the chlorophyll content and dry weight. Thus, exogenous NO can be used to alleviate Pb toxicity in *Melissa*. In fact, the lower concentration of NO (100 μ M) had a higher protective effect on Pb toxicity, while high concentration of NO (200 μ M) did not alleviate Pb toxicity considerably. NO may help plants to survive stressful conditions through its function as a signaling molecule in the activation of antioxidative enzymes or its direct reaction with active oxygen, nitrogen, and lipid radicals. While supplementation of SNP resulted in growth enhancement, as well as increase in the contents and activities of all investigated biochemical components. Thus, our results indicate that SNP application might alleviate Pb toxicities and regulate plant growth and development of *Melissa officinalis* and that Pb tolerance of *Melissa officinalis* cultivated in Pb-mediated nutrient solution increased at low concentrations of NO. This indicates that NO acts as an efficient ROS scavenger and membrane stabilizer in *Melissa officinalis* plants exposed to Pb stress.

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