The Roles of Selenium in Protecting Lemon Balm against Salt Stress

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Abstract

Plant metabolism and productivity is influenced adversely by salinity. Exogenous selenium (Se), applied as sodium selenate in biofortification programmes, has been found effective in alleviating the salt induced damage in plants. The study was conducted in order to determine the effects of exogenous Se supply (10 µM) on the resistance of lemon balm (*Melissa officinalis* L.) plants to salt stress (40 mM NaCl). Plant growth was negatively affected by salinity and dry mass production as well as chlorophyll *a* and *b* accumulation severely reduced. Selenium significantly improved the growth rate and increased the photosynthetic pigments and total amino acid contents in lemon balm plants subjected to salt stress. Salinity stress caused great membrane damage, as assessed by lipid peroxidation, but Se application significantly reduced the membrane damage because of an efficient scavenging by peroxidases (POD) and glutathione peroxidase (GSH-Px). Compared with the non-selenium treatment, application of Se increased the activity of phenylalanine ammonia-lyase (PAL) under salinity. As a result, the physiological and biochemical parameters measured in this study indicated that the salinity had adverse effects on growth of lemon balm plants, but the data also showed that presence of exogenous Se in nutrient solution could alleviate seedling damage under high levels of NaCl in the medium.

Keywords: Lemon balm; lipid peroxidation; photosynthetic pigments; medium; sodium selenate; salinity

*Abbreviation***:** APX: ascorbate peroxidase; CAT: catalase; DM: dry mass; FM: fresh mass; GSH-Px: glutathione peroxidase; H2O2: hydrogen peroxide; MDA: malondialdehyde; PAL: phenylalanine ammonia-lyase; POD: peroxidases; PVPP: polyvinylpolypyrrolidon; ROS: reactive oxygen species; Se: selenium; SOD: superoxide dismutase .

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Introduction

Plants are often subjected to various stress conditions such as high light intensity, UV radiation, temperature extremes, drought and salinity. A wide spectrum of biochemical and metabolic adaptations are found in plants under these stress conditions. Salinity reduces the availability of atmospheric CO2 because stomatal closure is increased and then decreases the consuming of NADPH by the Calvin cycle, which initiates chain reactions that produce more harmful oxygen radicals (Habibi, 2014). Accumulation of reactive oxygen species (ROS) damages critical organelles via lipid peroxidation

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and is capable of inducing damage to almost all cellular macromolecules including DNA, proteins and carbohydrates (Miller et al., 2010; Ding et al., 2010).

Activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POD) as the most important components in scavenging and prevention of ROS damage is increased under salt stress conditions (Dat et al., 2000). Moreover, a correlation exists between activity of antioxidant enzymes and salt tolerance of plants (Parida and Das, 2005).

Suitable plant nutrition is one of the strategies to avoid oxidative damage to cells (Kong et al., 2005). Recent researches have demonstrated that Se not only is able to promote growth and development of plants but also increases resistance and antioxidant capacity of plants subjected to various stresses (Feng et al. 2013). Selenium has been found to counteract the adverse effects of certain abiotic stresses, such as drought (Hasanuzzaman and Fujita, 2011), salt (Hasanuzzaman et al., 2011), cold (Chu et al., 2010) and high temperature (Djanaguiraman et al., 2010). The protective role of low Se concentrations in plants exposed to stress conditions in most cases has been attributed to increase antioxidant activity that can stimulate plant growth (Hussain et al., 2008).

Ozturk et al. (2004) reported that essential oil ratio of lemon balm (*Melissa officinalis* L.) plants, as a moderate salt-sensitive crop, was affected negatively by increasing salt concentration. According to this fact that the yield of lemon balm was reduced due to salinity stress, the understanding of the physiological and biochemical mechanisms conferring salt tolerance of this species is very important.

There is no information about the physiological responses of the lemon balm to selenium under salt stress, which may increase salinity tolerance. The present research was designed to examine if the presence of exogenous Se in nutrient solution could enhance resistance of *Melissa officinalis* L. against NaCl toxicity.

Materials and Methods

Plant Growth and Treatments

Seeds of *Melissa officinalis* L. were surface sterilized and germinated on filter paper moistened with distilled water and $CaSO_4$ at 0.05 mM. Ten-day-old seedlings were transferred to Hoagland nutrient solution (Johnson et al., 1957) and were pre-cultured for 35 days prior to the start of treatments. Plants were treated with nutrient solution containing: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM H_2MoO_4 and 0.02 mM $FeSO_4$-EDTA. The nutrient solution (pH 5.5–5.8) was renewed every 5 days. At 35 days after germination, the selenium (10 µM) and NaCl (40 mM) were applied together with the nutrient solution described above. Plants were grown under day/night temperature of 20-25/17-19 °C, relative humidity of 55-65 % and daily photon flux density of about 800-1000 µmol m^{-2} s^{-1} throughout the experimental period. At the end of the experiment (25 days after treatments), measurements were done and the recent fully expanded leaves were collected and frozen in liquid N_2 immediately until analysis.

Analysis of Growth Parameters

Leaves and roots were washed with distilled water, blotted dry on filter paper and after determination of fresh mass (FM) were dried for 48 h at 70 °C for determination of dry mass (DM).

Assay of Antioxidative Enzymes Activity and Related Metabolites

Activity of superoxide dismutase (SOD) and peroxidase (POD) was determined according to methods described elsewhere (Habibi and Hajiboland, 2010). For the determination of SOD activity, enzyme was extracted in 28 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture contained 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 13 µM riboflavin. One unit of SOD was defined as the amount of enzyme which produced a 50 % inhibition of NBT reduction under assay conditions. Peroxidase activity was determined using the guaiacol test at 470 nm. The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. The glutathione peroxidase (GSH-Px) activity was measured by a modification of the method of

Flohé and Günzler (1984) using the H_2O_2 as substrate. Enzyme was extracted in 50 mM phosphate buffer pH 7.0 and the supernatant was added to the reaction mixture contained 0.2 ml of the supernatant, 0.4 ml GSH (0.1 mM) and 0.2 ml $KNaHPO_4$ (0.067 M). The above reagents without supernatant extract were used for the non-enzyme reaction. After preheating the mixture on water bath at 25 °C for 5 min, 0.2 ml H_2O_2 (1.3 mM) was added to initiate the reaction. The reaction was stopped by adding 1 ml 1 % trichloroacetic acid and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 1100 g, 0.48 ml the supernatant was placed into a cuvette and 2.2 ml of 0.32 M Na_2HPO_4 and 0.32 ml of 1.0 mM DNTB were added for colour development. The absorbance at wavelength 412 nm was measured after 5 min. The enzyme activity was calculated as a decrease in GSH within the reaction time when compared with that in the non-enzyme reaction.

To assay for PAL activity, leaf samples were ground in 50 mM sodium phosphate buffer (pH 7.0) containing 2 % (w/v) polyvinylpolypyrrolidon (PVPP), 2 mM EDTA, 18 mM β-mercaptoetanol and 0.1 % (v/v) Triton X-100. After centrifugation (15000 g for 15 min at 4 °C), PAL was assayed in the supernatant by measuring the formation of cinnamic acid at 290 nm according to modified method of Zucker (1965): enzyme extracts were incubated at 30 °C for 60 min with 5 mM L-phenylalanine in 60 mM sodium borate buffer (pH 8.8). One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol cinnamic acid per h.

Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid. The hydrogen peroxide (H_2O_2) contents in the leaves were assayed according to the method of Velikova et al. (2000). Leaves were homogenized in ice bath with 0.1 % (w/v) TCA. The extract was centrifuged at 12,000 × g for 15 min, and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The reaction was improved for 1 h in the dark and measured spectrophotometrically at 390 nm. The content of H_2O_2 was given on a standard curve.

Determination of Chlorophylls and Total Free Amino Acids

Leaf concentration of chlorophyll *a*, *b* and carotenoids was determined after extraction of pigments in the cold acetone and allowing the samples to stand for 24 h in the dark at 4 °C (Lichtenthaler and Wellburn, 1985). Content of total free α-amino acids was assayed using ninhydrin colorimetric method. Leaf samples were ground in 50 mM sodium phosphate buffer (pH 6.8). After centrifugation (18,000 g for 15 min at 4 °C), ninhydrin reagent was added to sample solution and was incubated for 7 min at 80-100 °C in a water bath. After cooling, the absorbance was recorded at 570 nm. Glycine was used for production of standard curve (Hwang and Ederer, 1975). Total soluble proteins were determined spectrophotometrically using a commercial reagent (Bradford reagent, Sigma) and bovine albumin serum (BSA) as standard (Bradford, 1976).

Experiments were undertaken in complete randomized block design. All experiments were conducted using 4 independent replications. Statistical analyses were carried out using sigma stat (3.5) with Tukey test ($P < 0.05$).

Results

Plants treated with 40 mM NaCl showed the highest level of stress leading to lower shoot matter values during the time period studied (Fig. I.). However, exogenous Se applied to plants caused higher shoot dry matter when compared to those without application of Se under salinity conditions. Salt stress also remarkably decreased the root dry weight. Selenium significantly improved the root growth of lemon balm that was grown under NaCl-stressed conditions. The content of chlorophyll *a* and *b* was significantly decreased by salt stress, and the decrease in the Se-supplied plants was obviously less than that of the non-supplied plants (Table 1). The content of carotenoids was not influenced significantly by salt stress and Se application.

An increase in the amount of total amino acids was observed under salt stress conditions compared with control (Fig. II.). In contrast, when selenium was applied together with salinity, the root amino acid content was increased

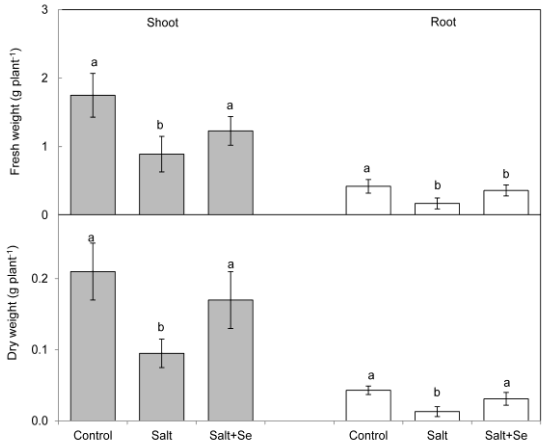

Fig. I. Effects of application on dry and fresh mass of lemon balm under salinity conditions. Bars with the same letter within each shoot (colored) and root (non-colored) are not significantly different (P < 0.05). Values are the mean ± SD (n = 4).

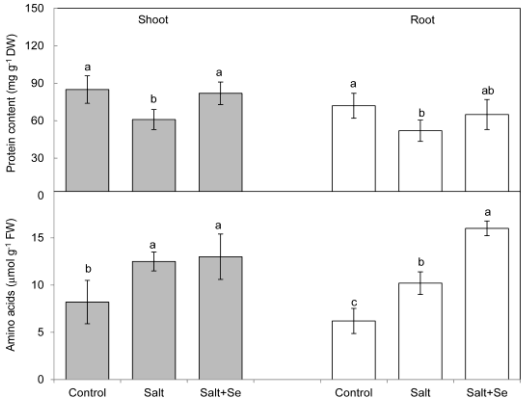

Fig. II. Effects of Se application on the concentration of total amino acids and proteins. Bars with the same letter within each shoot (colored) and root (non-colored) are not significantly different (P < 0.05). Values are the mean ± SD (n = 4).

Table 1.
Effect of Se supplementation on the concentration of chlorophylls *a*, *b* and carotenoids in lemon balm plants grown for 25 days under salt stress conditions. Data of each parameter by the same letter are not significantly different (P < 0.05). Values are the mean ± SD (n = 4).

Treatments	Chlorophyll *a* (mg g^{-1} FM)	Chlorophyll *b* (mg g^{-1} FM)	Carotenoids (mg g^{-1} FM)
Control	0.30 ± 0.05 ^{a}	0.14 ± 0.02 ^{a}	0.09 ± 0.02 ^{a}
Salt	0.06 ± 0.03 ^{c}	0.05 ± 0.04 ^{b}	0.08 ± 0.03 ^{a}
Salt+Se	0.16 ± 0.04 ^{b}	0.11 ± 0.01 ^{a}	0.09 ± 0.01 ^{a}

significantly. At the end of the experiment, leaf

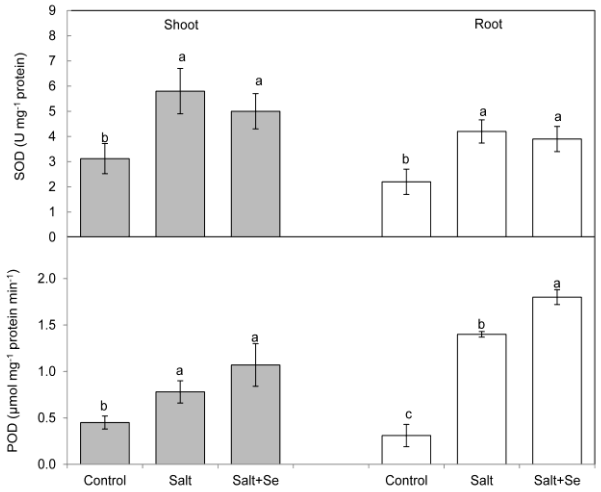

Fig. III. Effects of Se application on the concentration of total amino acids and proteins. Bars with the same letter within each shoot (colored) and root (non-colored) are not significantly different ($P < 0.05$). Values are the mean ± SD (n = 4).

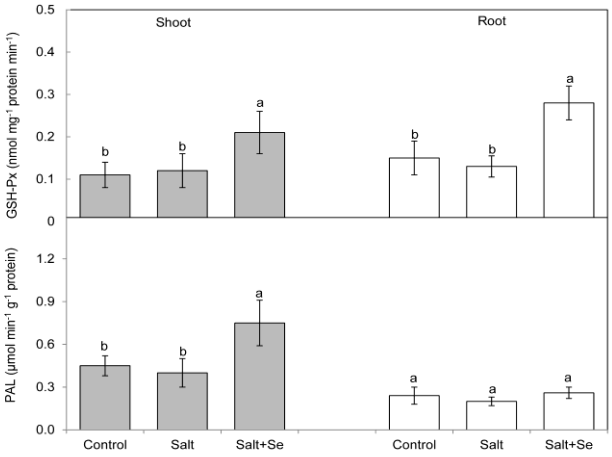

Fig. IV. Effects of Se application on the activity of GSH-Px and PAL. Bars with the same letter within each shoot (colored) and root (non-colored) are not significantly different ($P < 0.05$). Values are the mean ± SD (n = 4).

protein content decreased by 20 days salt stress, in comparison to plants under non-saline conditions. However, exogenous Se ameliorated protein reduction of salt-stressed *Melissa officinalis* plants.

In shoots, the activity of PAL in stressed plants without Se was not influenced, and application of selenium increased the activity of this enzyme under salinity conditions. The activity of PAL was not influenced significantly by salt stress and Se application in the roots.

In this study, an increase in the activity of SOD was observed under salinity conditions. In addition, activity of SOD was also increased by Se application in salt-stressed plants. In the present work, the shoot POD activity was significantly

increased by salinity, but there was no significant difference between the ‘‘Salt’’ and ‘‘Salt+Se’’ treatments. As shown in Fig. III, the POD activity in the roots was increased in the presence of Se. In *Melissa officinalis* shoots and roots, continuation of the salt stress was not significantly influenced the GSH-Px activity, but Se application caused a significant increase in the activity of this enzyme (Fig.IV).

observed a substantial decrease in the shoot matter by about 50 % compared with control.

Although, a significantly rise in the shoot matter in the Se-supplemented NaCl-stressed samples relative to NaCl-stressed treatment revealed that Se exerts beneficial effects on the growth of *Melissa officinalis* plants. In fact, these data are similar to those reported by Kong et al. (2005), who suggested that an appropriate

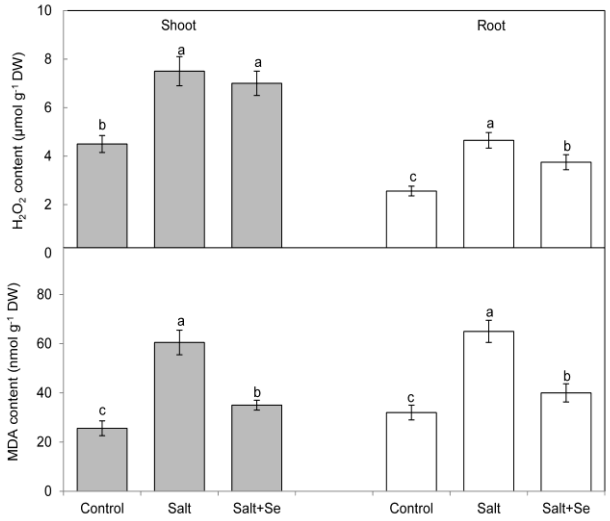

Fig. V. Effects of Se application on the concentration of MDA and H_2O_2. Bars with *the* same letter within each shoot (colored) and root (non-colored) are not significantly different ($P < 0.05$). Values are the mean ± SD (n = 4).

In this research, salt stress without Se application caused significant accumulation of hydrogen peroxide (H_2O_2) and malondoialdehyde (MDA) in *Melissa officinalis* shoots and roots (Fig. V). However, Se supplemented plants maintained lower MDA content under salt stress. In shoot samples, continuation of the salt stress with or without selenium application caused significant accumulation of H_2O_2 content. The hydrogen peroxide level was decreased by Se application under salt stress in the root samples.

Discussion

Among plant species, *Melissa officinalis* L. is considered to be moderate salt-sensitive crop (Ozturk et al., 2004). Once moderate stress condition for the *Melissa officinalis* physiological apparatus was defined (40 mM NaCl); we

concentration of exogenous Se functions positively to promote the osmoregulatory capacity, and increases the growth.

The reduction of chlorophyll concentration in salt-stressed plants could be attributed to the inhibition of chlorophyll biosynthesis following an increase in ethylene production (Sultana et al., 1999). However, exogenous Se ameliorated chlorophyll reduction of salt-stressed *Melissa officinalis* plants. These results are in agreement with other findings showed that application of Se increased chlorophyll *a* concentration (Chen et al., 2008).

Osmotic adjustment often happens as a result of the accumulation of osmolytes such as amino acids and minerals (Sonobe et al., 2011). In this study, an increase in the amount of total free amino acid was observed under stress conditions compared with controls, but this was more

increased by Se treatment under salinity stress in *Melissa officinalis* roots and this increase was accompanied by an increased root water uptake.

Additionally, results from this study revealed that the PAL activity was increased by supplementary Se. The protective role of Se in salt-stressed plants may be related to activation of PAL which is responsible for synthesis of phenolic compounds. This finding was in accordance with Walaa et al. (2010), who observed that Se application significantly promoted the specific activity of PAL.

Like other abiotic stress conditions, the salinity effect also comprises accumulation of ROS which lead to oxidative stress of plants (Hasanuzzaman et al. 2011). Enhancement of antioxidative enzymes activities is one of the mechanisms participating in the protection from ROS hyperaccumulation. Therefore, a significantly rise in the activity of POD and GSH-Px revealed that Se exerts beneficial effects on stress tolerance of *Melissa officinalis* by enhancing their antioxidative capacity. In this respect, Hasanuzzaman et al. (2011) found stronger antioxidant enzymes activities in Se-supplemented rapeseed plants under saline stress. Results from this study revealed that the amounts of MDA remained unchanged under Se-supplemented NaCl-stressed conditions obviously because of an efficient scavenging following significant enhancement of POD and GSH-Px activities. Several studies have shown that a protective role of Se against the oxidative stress in higher plants coincided with enhanced GSH-Px activity and decreased lipid peroxidation (Cartes et al., 2005; Habibi, 2013). As a result, Se-supplemented NaCl-stressed plants exhibit better protection from salt-stressed damage because of higher POD and GSH-Px activities and lower levels of H_2O_2 and MDA as compared to salt stress alone, similarly with that observed in Se-supplemented NaCl-stressed rape seedlings (Hasanuzzaman and Fujita, 2011).

The results indicate that under salt stress, addition of Se can increase growth as well as photosynthetic pigments in NaCl-treated seedlings. Furthermore, selenium applied plants maintained lower MDA content, which indicated that application of Se could prevent lipid peroxidation of stressed lemon balm plants obviously because of an efficient scavenging of ROS following elevated activity of POD and GSH-Px. In addition, Se treatments at 10 µM significantly improved the specific activity of PAL in lemon balm leaves subjected to 40 mM NaCl. However further research is needed to solve the nature of this efficacy. Finally, this research shows that Se application can be useful to promote salinity resistance in lemon balm plants. These results are the first that evidence the protective effect of Se in lemon balm plants under salt-stressed conditions. Further studies are necessary to better understand how Se acts in lemon balm plants subjected to salt stress.

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