

## Green synthesis of Se nanoparticles and its effect on salt tolerance of barley plants

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### Abstract

In this study, selenite ions were reduced to selenium nanoparticles using a leaf extract of barley (*Hordeum vulgare L.*) plants. Characterization of synthesized nanoparticles using Scanning Electron Microscopy (SEM) and UV-visible spectrophotometry indicated the formation of variable size of selenium nanoparticles, suggesting that leaf extract could form polydispersed nanoparticles. Then we used these synthesized selenium nanoparticles to mitigate salt stress in barley plants under hydroponic conditions. Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) analyses suggested that the hydroponically nano-Se application resulted in direct accumulation of Se in the leaves of barley. Shoot growth was negatively affected by salinity levels up to 100 mM, whereas this reduction was mitigated by application of exogenous Se nanoparticles. Our results indicated that high salinity stress decreased the activity of superoxide dismutase (SOD), and enhanced the levels of malondialdehyde (MDA) in the leaves of barley seedlings, whereas application of Se nanoparticles increased total phenolic levels, and also resulted in a significant reduction of MDA (a marker for the ROS-mediated cell membrane damage) contents, which could influence the metabolism and be responsible for the increasing shoot dry weight. These results provided the first evidence that the green Se nanoparticles promote the growth of barley seedlings under salt stress.

**Keywords:** Antioxidant Defense System; Green Nano-Se; *Hordeum vulgare L.*; Malondialdehyde; Salt Stress; Shoot Growth.

### How to cite this article

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### INTRODUCTION

Selenium (Se) is not essential for higher plants [1], however, it exerts diverse beneficial effects on plant growth at low concentrations [2, 3], perhaps by enhancing their antioxidative capacity [4, 5], and increasing plant resistance against oxidative stress [6, 7]. Exogenous application of Se at low levels exhibits the capacity to enhance the plant growth and yield as well as stress tolerance under salinity [4] by inhibition of membrane lipids peroxidation and enhancing the activities of antioxidant enzymes [8].

Today, nano-Se has been introduced as highly stable nano-elemental selenium for use in nutritional supplements for applications in medical therapy [9] as well as Se fertilization for increasing crop yield and agricultural productivity

and food security [10]. The biological activity of these Se nanoparticles depends on its size [11]. Several routes have been developed for synthesis of nanoparticles including various physicochemical methods [12] and biogenic synthesis methods [13]. Compared with some of the physicochemical production methods, biological or biogenic synthesis of nanoparticles from salts of the corresponding metals is free of contamination and has a well-defined size and morphology [14].

Different genus *Bacillus cereus* [15], *Bacillus subtilis* [16], and a variety of fungi [17] have been exploited to produce nanoparticles. Many studies have been published concerning the different biological effects of nano-Se on plants including rice ([18], tobacco [19], tomato [20] and giant reed [21]. In contrary, a few studies have been published concerning the biosynthesis of Se

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nanoparticles using plant extracts [10]. Recently, Prasad et al. [22] used an aqueous leaf extract of lemon plant for synthesis of colloidal selenium nanoparticles. In the present work, we used an aqueous leaf extract of barley plant as a precursor for synthesis of selenium nanoparticles. Most research has focused on assessing the nature of the phytotoxicity of nanoparticles, but quantitative methods for determining nanoparticles in plant tissues have not been considered. Therefore, in this study, uptake and accumulation of green-synthesized Se nanoparticles were quantified by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) analyses. Se nanoparticle has a high biological activity and low toxicity [23]. Regarding to the best of our knowledge, the reports concerning the effect of green-synthesized Se nanoparticles on plants are not existent. To address this issue, we examined in some detail the biochemical mechanisms by which nano-Se affects the photosynthetic pigments, phenolic compounds and antioxidant capacity. Since the elemental Se mitigates salt-induced membrane damages resulting in better plant growth under salt stress, we hypothesize that nano-Se can also alleviate salt-induced damages.

## MATERIALS AND METHODS

### *Synthesis of selenium nanoparticles and characterization*

Fresh leaves (5 g) of barley plant were digested with 20 ml of Tris-Cl (pH 7.5). After centrifugation at 10,000g for 5 min at 4 °C, supernatant was used as a precursor for synthesis of selenium nanoparticles. The leaf extract (2 ml) containing dissolved phytochemicals was added drop wise in to 20 ml of sodium selenite solution (10 mM) under magnetic stirring. The synthesis of selenium nanoparticles was determined by sampling an aliquot (3 ml) of the mixture at intervals of 12 h, followed by measurement of the UV-vis spectra using spectrophotometer according to the method described in Prasad et al. [22].

To estimate the absorption maximum, a spectral scanning analysis was performed by measuring optical density of the content from wavelength, 200 to 800 nm. The leaf extract containing Se nanoparticles were subjected to centrifugation at 10,000 rpm for 10 min. To remove possible organic contamination present in nanoparticles, the pellet was successively washed by centrifugation 10,000 rpm for 10 min with de-

ionized water according to Prasad et al. [22] and finally, pellet was freeze dried using a lyophilizer. The powder of synthesized nanoparticles obtained in this manner was subjected to visualize using a scanning electron microscope (SEM, Seron Technology, AIS2100 model).

To determine the synthesized-nanoparticle concentration, 100 µL of each nanoparticle solution was digested in 400 µL of a mixture of HNO<sub>3</sub> and HCl (v/v, 3 : 1). Then, the digested solution was diluted with 4.5 mL 1% nitric acid according to the method described in Raliya et al. [24]. Elemental concentration of Se in each sample was determined using Inductively-Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, INTEGRA XL2, GBC; Australia)

### *Growth conditions and exposure of nanoparticles to plant*

Seeds of Barley (*Hordeum vulgare* L.) were surface sterilized and germinated on filter paper moistened with distilled water. Ten-day-old seedlings were transported to modified Hoagland nutrient solution [25] containing 6 mM KNO<sub>3</sub>, 4 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 50 µM H<sub>3</sub>BO<sub>3</sub>, 2 µM MnSO<sub>4</sub>, 2 µM ZnSO<sub>4</sub>, 0.5 µM CuSO<sub>4</sub>, 0.5 µM H<sub>2</sub>MoO<sub>4</sub> and 0.02 mM FeSO<sub>4</sub>-EDTA for 25 days prior to the start of treatments. Selenium nanoparticle sample was diluted with DI water to 100 ppm for treatment. At 25 days after germination, Se nanoparticles (0 or 100 ppm) and NaCl (0, 100 or 200 mM) were applied together with the nutrient solution described above. Plants were grown with a temperature regime of 22-25/17-19 °C, relative humidity of 60-65 % and daily photon flux density of about 300-350 µmol m<sup>-2</sup> s<sup>-1</sup> throughout the experimental period.

### *Harvest plants*

Plants were harvested 14 days after applying the nanoparticles. Fully expanded and mature leaves were used for measurement of enzymatic analysis. Shoots and roots were washed with distilled water, blotted dry on filter paper and after determination of fresh weight (FW) they were dried for 48 h at 70 °C for determination of dry weight (DW). Plants height and tap root length were measured using a ruler.

### *Analysis of selenium*

The total Se content was measured according to the method reported by Liu and Gu [26]. 1 g of

the sample was digested with 5 ml of a mixture of  $\text{HNO}_3$  and  $\text{HClO}_4$  (v/v, 4:1) at 130 °C for 1 h. After cooling, 5 ml of concentrated HCl was added and incubated at 115°C for 20 min. Then, the digested solution was used for total Se determination by Inductively-Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, INTEGRA XL2; GBC).

#### *Determination of total carotenoids and chlorophylls a and b*

Leaf concentration of chlorophyll and carotenoids was calculated after extraction of pigments in the cold acetone and allowing the samples to stand for 24 h in the dark at 4 °C [27].

#### *Assay of antioxidative enzymes and related metabolites*

The activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were estimated according to methods described elsewhere [28]. For determination of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) contents in the leaves, samples were homogenized in ice bath with 0.1% (w/v) TCA, after centrifugation at 12,000g for 15 min, 0.5 ml of the supernatant was added 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 according to the method described in [29]. The content of  $\text{H}_2\text{O}_2$  was given on a standard curve. Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid according to methods described elsewhere [28].

#### *Assay of phenylalanine ammonia-lyase (PAL) activity and related metabolites*

PAL activity was extracted and determined by the modified method of Zucker [30] via measuring the formation of cinnamic acid at 290 nm. The method described in Velioglu *et al.* [31] was used for determination of total phenolic content. Gallic acid was used for the production of standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight. Total flavonoid content was estimated using the method adapted by Meda *et al.* [32]. Briefly, 5 ml of 2% aluminium chloride ( $\text{AlCl}_3$ ) in methanol was mixed with the same volume of leaf extracts (0.02 mg/ml). Absorption readings at 415 nm were taken after

10 minutes against a blank sample without  $\text{AlCl}_3$ . Quercetin (Sigma) was used for production of a standard curve. The total flavonoid content was expressed as mg quercetin equivalent (QE)/100 g extract.

#### *Nanoparticle uptake and accumulation analysis using ICP-MS*

After nanoparticle exposure to plants hydroponically, plants were allowed to grow for 48h in the environmental condition described above. Cellular uptake and accumulation of Se nanoparticles were quantified by Inductively-Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, INTEGRA XL2, GBC; Australia).

#### *Statistical analyses*

Experiment was undertaken in complete randomized block design with 4 independent replications. Statistical analysis was carried out using ANOVA followed by Tukey test (Sigma Stat, 3.5; Systat Software Inc., San José, California). Results were given as mean  $\pm$  standard deviation (SD). Differences between treatments were considered to be significant, when a P value was less than 0.05 ( $P < 0.05$ ).

## **RESULTS AND DISCUSSION**

#### *Biosynthesis of Se nanoparticles using barley extract and characterization*

In this study, selenium ions could be reduced to nanoparticles using a leaf extract of barley plants. Initially the colloidal solution appeared colorless (Fig. 1a) but after reduction of selenite during incubation period of 24 h, light red color was obtained (Fig. 1b). This change in color as well as building of absorbing maximum at 275 nm (Fig. 1c) can be attributed to the formation of selenium nanoparticles. SEM analysis of colloidal solution demonstrated the formation of selenium nanoparticles (Fig. 2). This analysis represented that size of particles generated using leaf extract ranges from 50 to 200 nm. In recent decades, literature demonstrated that plant extracts were used for plant mediated nanoparticle synthesis [33]. In this work, we synthesized selenium nanoparticles using leaf extracts of barley plants. Similarly, Prasad *et al.* [22] produced colloidal selenium nanoparticles with anti DNA damaging property using aqueous leaf extract of lemon plant.

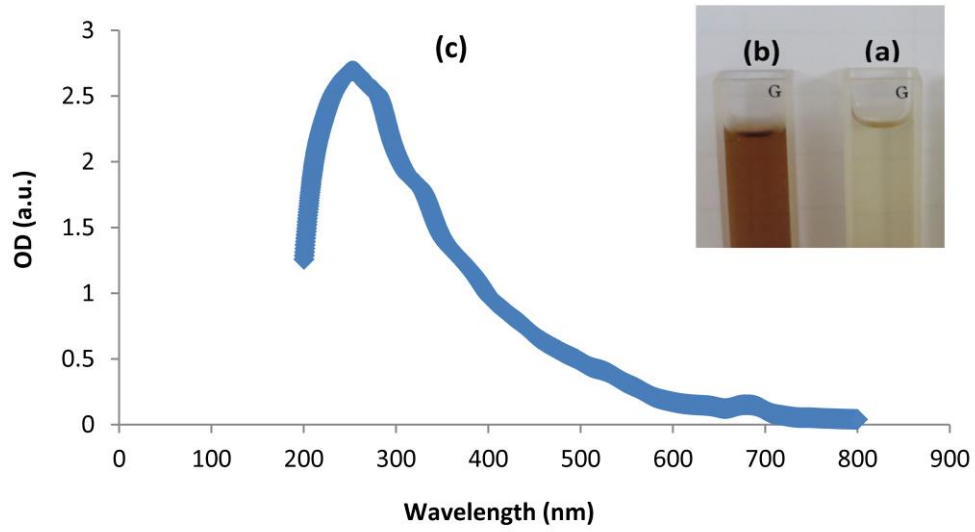


Fig. 1. A visible observation of change in color during selenium nanoparticle formation. A cuvette containing 10 mM of  $\text{Na}_2\text{SeO}_3$  solution, immediately after addition of barley plant leaf extract (a). A light red color appeared when selenium salt was mixed with extract followed by incubation for 24 h (b). UV-Vis spectra of selenium nanoparticles synthesized using barley leaf extract (c).

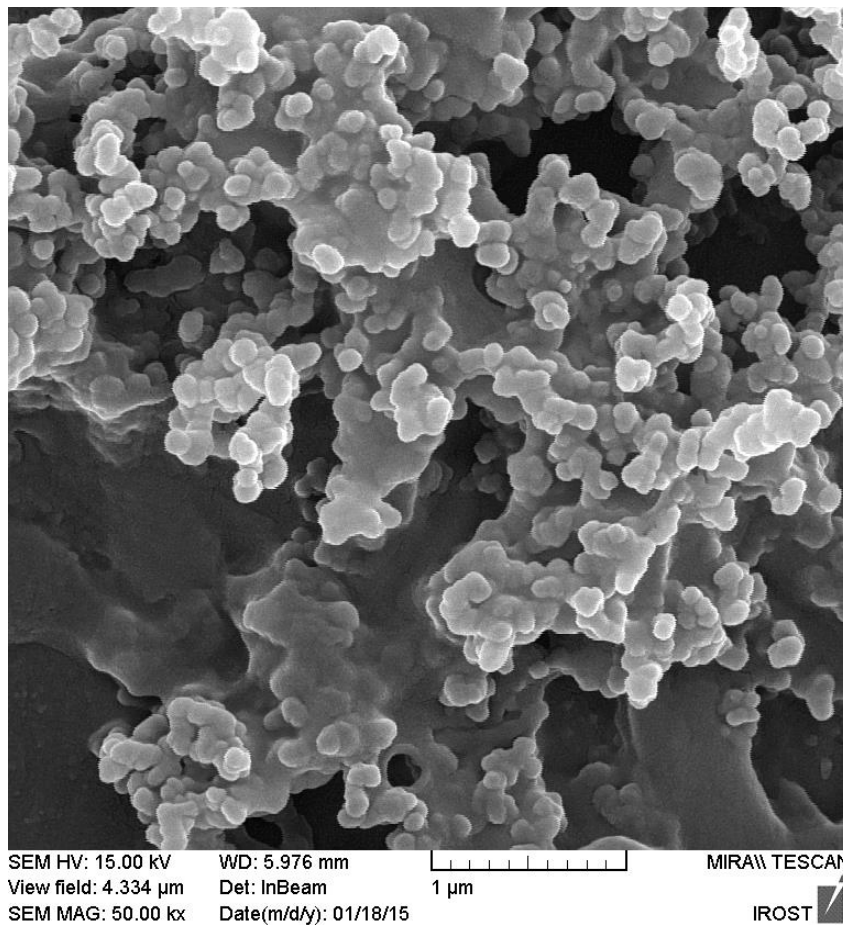


Fig. 2. Scanning electron microscopy image of Se nanoparticles prepared using barley leaf extract.

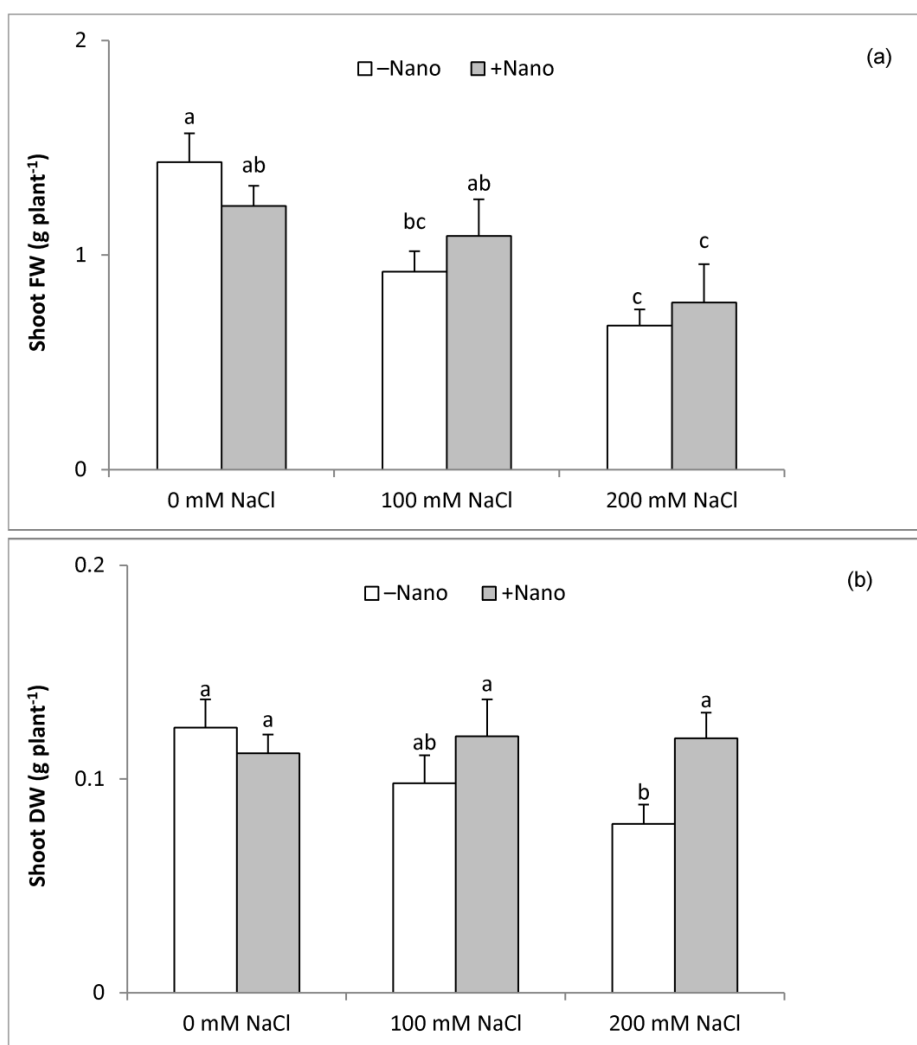


Fig. 3. Effects of Se nanoparticles application on the shoot fresh (a) and dry weight (b) of barley seedlings exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

#### *Se-nano pretreatment improved shoot growth during salt stress*

Since Se nanoparticles have an excellent bioavailability and low toxicity for increasing crop yield and agricultural productivity [22], we used green Se nanoparticles to mitigate stress in barley plants treated with salinity. Shoot growth was negatively affected by salinity levels up to 100 mM (Fig. 3a, b). This reduction of shoot dry weight was mitigated by application of exogenous Se nanoparticles. Similarly, Babajani *et al.* [3] showed that Se NPs led to a significant increase in *Melissa officinalis* biomass. Moreover, fresh weight of

shoots was lower under both mild (100 mM) and high (200 mM) salinity compared to control conditions. Fresh weight of roots was not negatively decreased even under the highest salinity levels applied (Fig. 4a). Only root dry weight was higher in Se-supplied plants at the lowest salinity level (100 mM) as compared to control plants (Fig. 4b). Shoot length was significantly decreased by salinity even at the lowest level applied (Fig. 5a). However root length was decreased only under high salinity (200 mM) (Fig. 5b). Moreover, shoot and root length of plants was not affected by exogenous nano-Se application during the experiment.

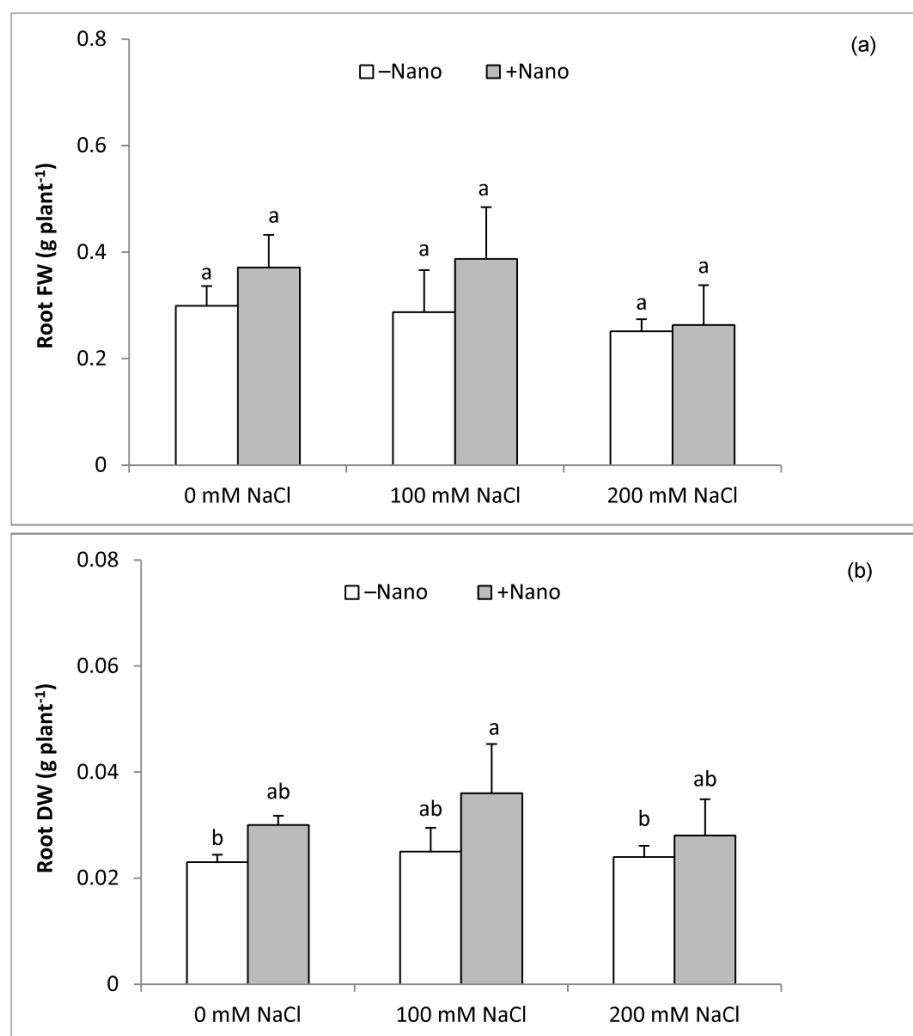


Fig. 4. Effects of Se nanoparticles application on the root fresh (a) and dry weight (b) of barley seedlings exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

Barley is a crop with relatively high tolerance to salt stress [34]. Where the salinity rises to 100 mM NaCl (about  $10 \text{ dS m}^{-1}$ ), barley exhibits a reduced yield. However, barley dies at salt concentrations higher than 250 mM NaCl (about  $25 \text{ dS m}^{-1}$ , or 50% seawater) [34]. The present study also supported this conclusion. Here, we showed that under high salinity (200 mM NaCl), the majority of growth parameters such as shoot dry weight, shoot and root length tended to decrease. However, enrichment of NaCl-containing medium with nano-Se resulted in significant increase in shoots biomass, when compared to NaCl-stressed plants grown without nano-Se addition. These results

suggested that nano-Se alleviates salt-induced oxidative stress. Despite few studies have been published concerning the effects of elemental Se on the performance of plants under salt stress [4, 35], information is lacking on the effect of nano-Se treatment on plants under salt stress. It has been shown that higher concentrations of nano-Se stimulate the organogenesis and the growth of root system significantly in tobacco [19]. Similarly, in this study, in nano-Se supplied seedlings under mild salinity (100 mM NaCl), the dry weight of roots even tended to increase.

Thus, our findings indicated that the hydroponically nano-Se application resulted in

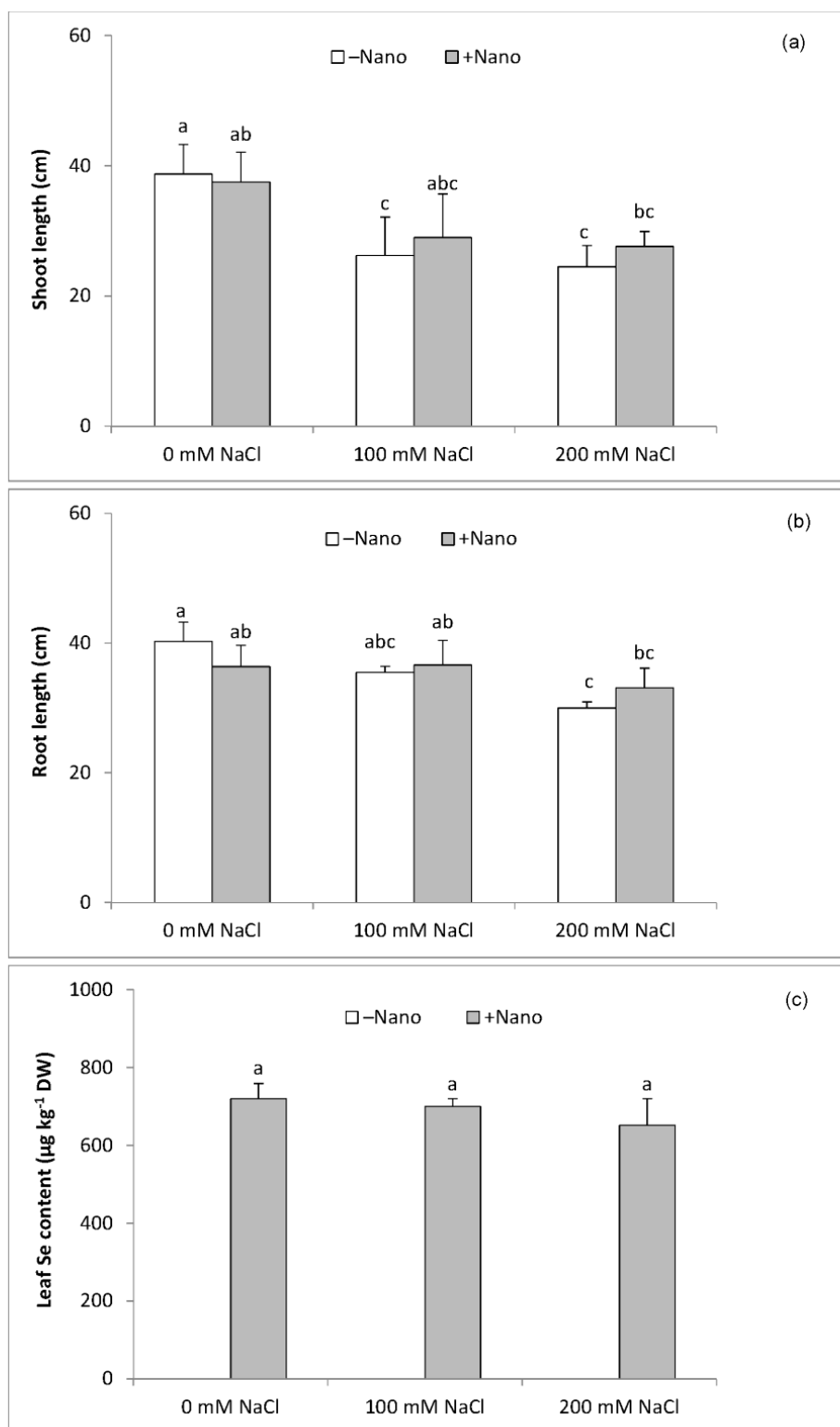


Fig. 5. Effects of Se nanoparticles application on the shoot (a) and root elongation (b), and Se accumulation (c) (recovered by ICP-MS) in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).



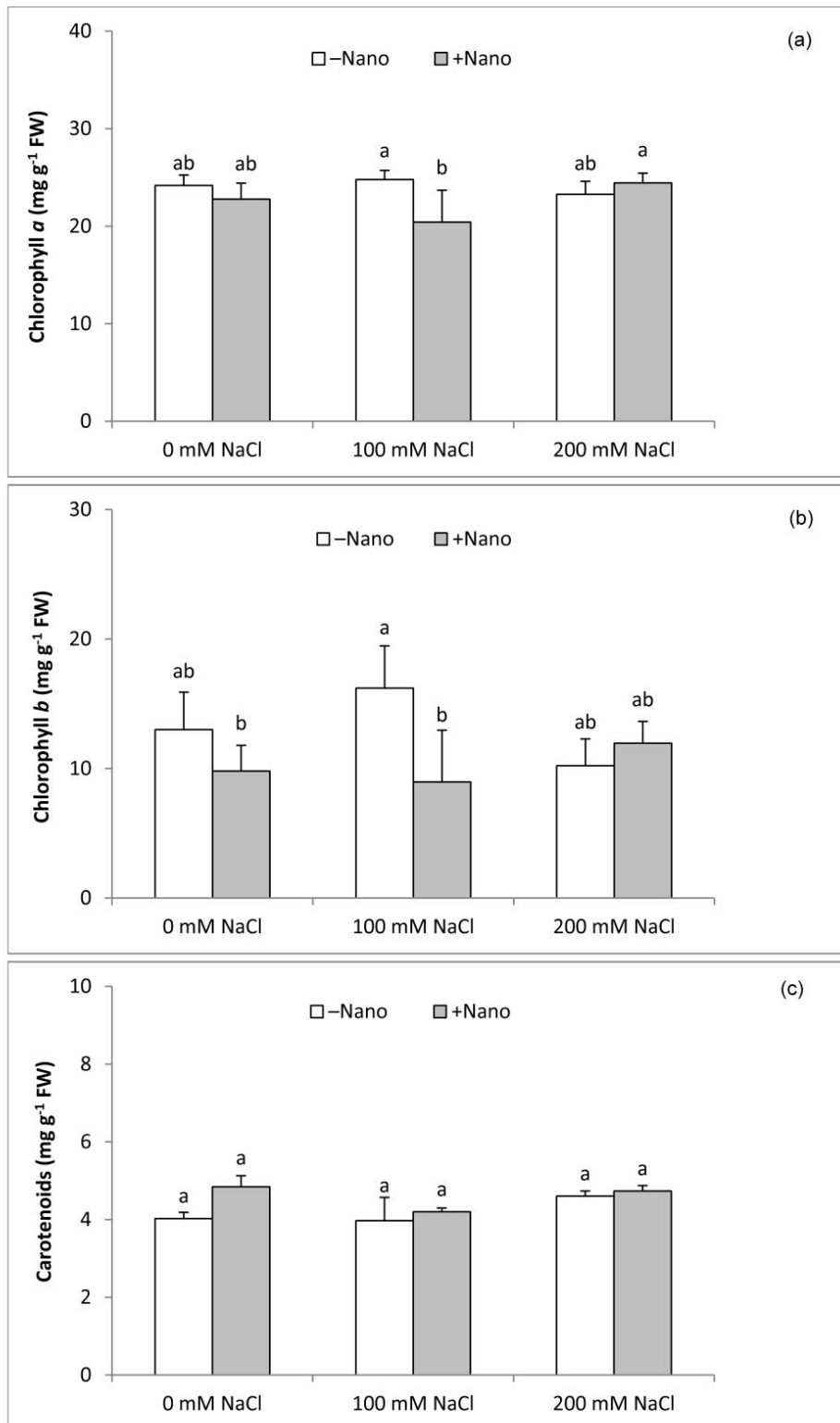


Fig. 6. Effects of Se nanoparticles application on the Chl *a* (a), *b* (b) and carotenoid (c) contents in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).



direct accumulation of Se in the leaves of barley, which mitigated the negative effects of salinity stress on barley growth.

#### *Nanoparticle uptake and accumulation analysis using ICP-MS*

We used green Se nanoparticles to mitigate salt stress in barley plants. To detect Se NPs concentration in leaves, we used ICP-MS analyses; and the measured concentration of elemental Se in leaf samples was normalized by the dried mass of the leaves. Exogenous nano-Se application increased endogenous Se contents in leaves of barley plants under non-saline and salt stress conditions (Fig. 5c). These results agreed with Domokos-Szabolcsy *et al.* [19] who reported that nano-Se was taken up by tobacco callus cultures and rooted tobacco plantlets and with Hu *et al.* [36] who reported absorption and bio-transformation of selenium nanoparticles by wheat seedlings. Indeed, most research have mainly focused on assessing the nature of the safety and toxicity of these nanoparticles [37, 38], but the uptake and entry of nanoparticles into plant systems is still poorly understood [38].

#### *Se-nano pretreatment did not change leaf photosynthetic pigments and flavonoids during salt stress*

Leaf photosynthetic parameters including chlorophyll and carotenoid contents were not significantly influenced under salt stress with or without nano-Se treatment (Fig. 6a, b and c). However, a consistent tendency of chlorophyll *b* to increase in response to low salinity was observed, but this increase was not significant (Fig. 6b). It has been reported that the application of selenium to the NaCl containing medium causes an increase in the concentration of chlorophylls and carotenoids [2]. Although, the photosynthetic pigment concentration was not influenced by NaCl and nano-Se treatments in this study. Interestingly, results showed that the nano-Se increased the leaf phenolic under high salinity conditions (Fig. 7a). Flavonoid concentration remained unchanged under salt stress with or without nano-Se treatment (Fig. 7b). Results from this study revealed that the PAL activity was increased by supplementary nano-Se and 100 mM NaCl (Fig. 7c). The higher phenolic accumulation can represent a mechanism that

increases the antioxidant capacity and radical-scavenging activity [39].

#### *Selenium nanoparticle was an alleviant for the oxidative stress effects caused by NaCl*

However, SOD enzyme did not respond to mild salinity levels, but a significant reduction of SOD activity was observed at 200 mM (Fig. 8a). The activity of CAT was not negatively influenced even under the highest salinity levels applied (Fig. 8b). However, the action of antioxidant enzymes are increase with the presence of Se in plants [40], a consistent tendency of CAT activity to decrease in response to nano-Se was observed, followed by increase under higher salinity conditions. In particular, under high salinity (200 mM), exogenous nano-Se application did not increase the activity of SOD and CAT in the leaves of barley plants, suggesting that other antioxidant mechanisms exist. Indeed, in the presence of Se,  $H_2O_2$  is primarily scavenged by glutathione peroxidase (GPX) instead of CAT, moreover, the higher activity of the ascorbate-glutathione cycle is one of the important mechanisms for Se to mitigate salt stress [4], while we did not measure those enzymes activity. On the other hand, there is no information about the antioxidant defense responses of the plants to nano-Se application under salt stress, which may increase salinity tolerance.

The occurrence of oxidative stress upon salt treatments was evaluated by the accumulation of MDA, a marker for the ROS-mediated cell membrane damage. In this study, a significant increase of  $H_2O_2$  and MDA concentrations was observed under salt stress (Fig. 9a, b); however, treatment of seedlings with nano-Se was effective in decreasing leaf MDA concentrations. Additionally, we observed that the contents of antioxidant metabolites such as phenolic compounds in the salt-stressed seedlings were enhanced by nano-Se application, which was consistent with the ability of Se to reduce the content of  $H_2O_2$  and MDA in this plant. Although several studies have been shown that Se increases growth promoting activities of higher plants via enhancing their antioxidative capacity against oxidative stress [5, 41], but the mechanisms for nano-Se to mitigate salt stress is still unknown and must be further explored.

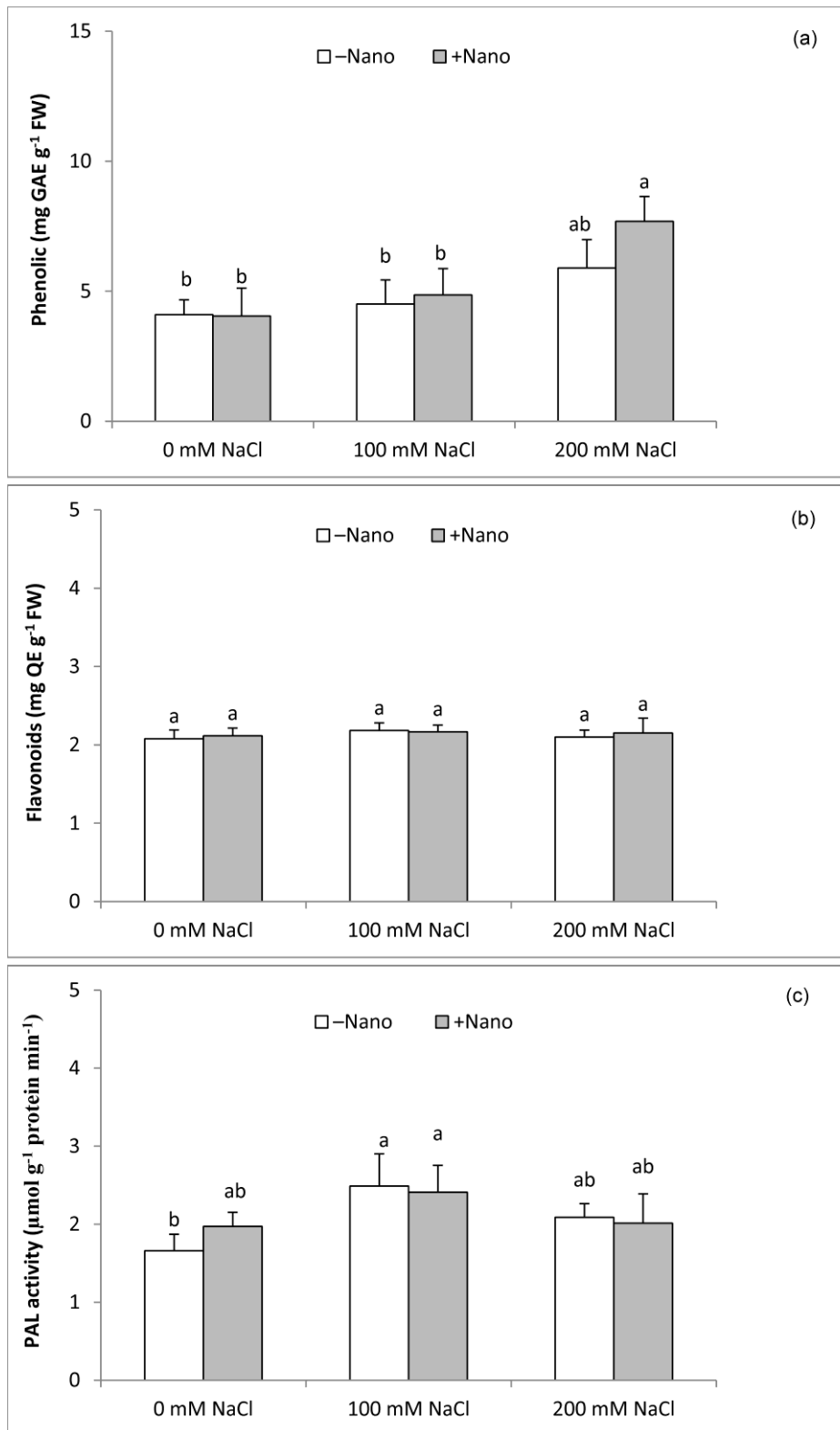


Fig. 7. Effects of Se nanoparticles application on the total phenolic (a) and flavonoid content (b), and specific activity of phenylalanine ammonia-lyase (PAL) (c) in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

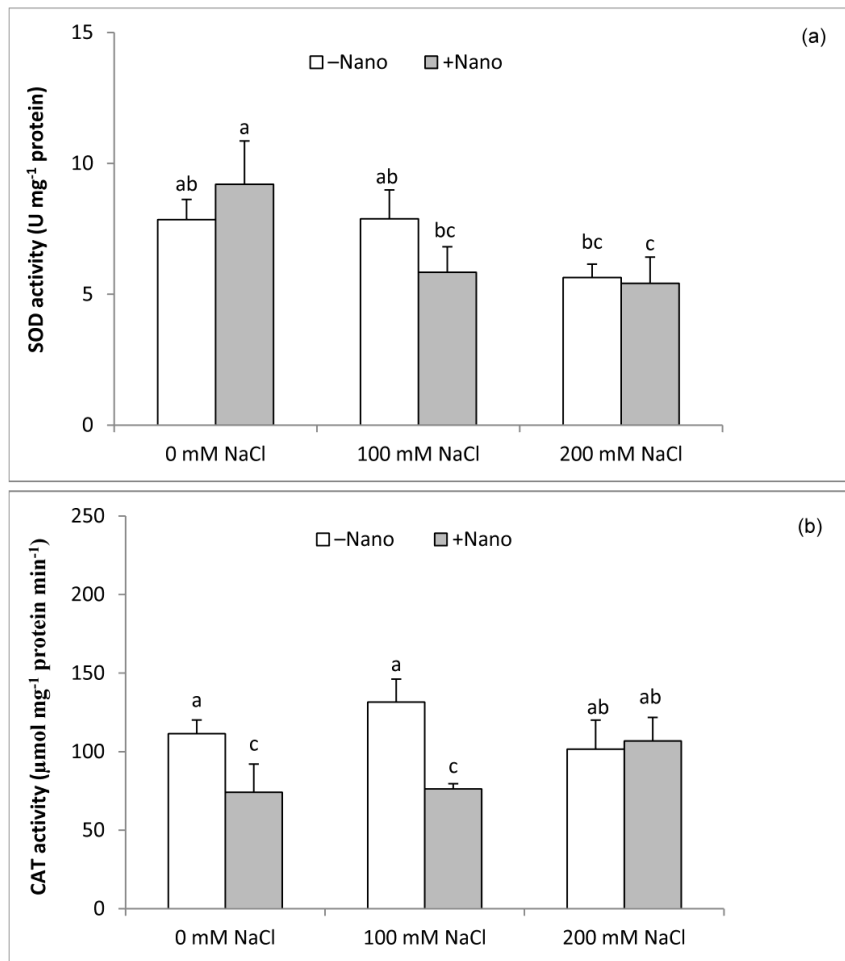


Fig. 8. Effects of Se nanoparticles application on the activity of superoxide dismutase (SOD) (a) and catalase (CAT) (b) in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

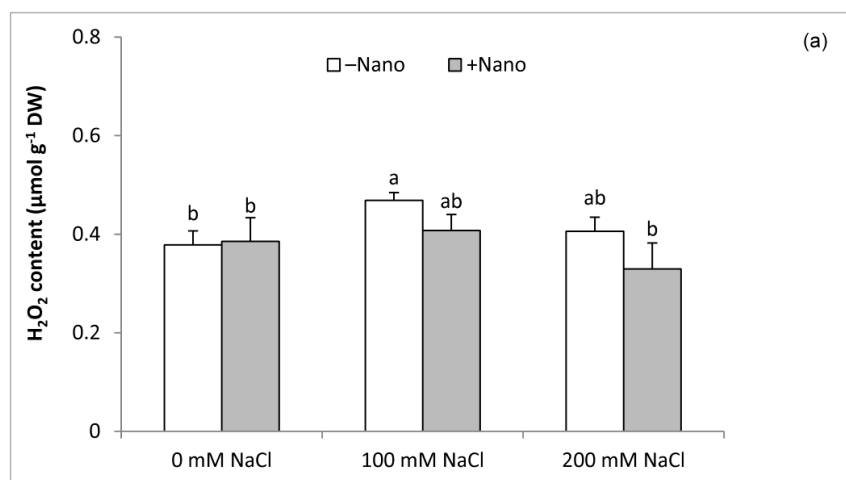


Fig. 9. Effects of Se nanoparticles application on the concentration of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (a) and malondialdehyde (MDA) (b) in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

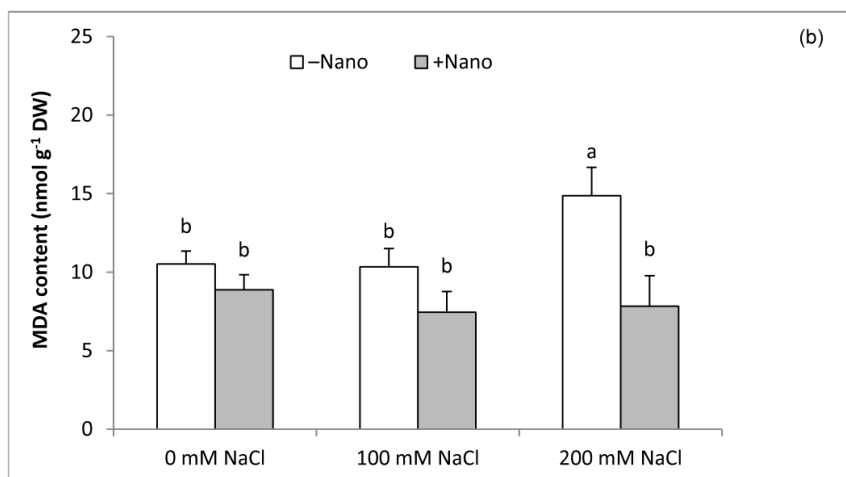


Fig. 9. Effects of Se nanoparticles application on the concentration of hydrogen peroxide ( $H_2O_2$ ) (a) and malondialdehyde (MDA) (b) in barley leaves exposed to NaCl (0, 100 or 200 mM) for 14 days. Error bars indicate the standard deviation. Bars indicated with the same letter are not significantly different ( $P < 0.05$ ).

## CONCLUSION

This is the first report of synthesis of Se nanoparticles using a leaf extract of barley plants. SEM results and UV-Vis spectrophotometry demonstrated the formation of selenium nanoparticles. Then we used these green nanoparticles to mitigate salt stress in barley plants under hydroponic conditions. Salt stress reduced shoot growth, and enhanced the levels of MDA in the leaves of barley seedlings; however, salt-induced negative effects were significantly diminished in the nano-Se-pretreated plants. In the present study, we concluded that nano-Se ameliorates the negative effect of high salinity on productivity without any symptoms of Se toxicity in plants. Nevertheless, further research is needed for more details concerning different effects of nano-Se on plant nutrition and antioxidant defense system.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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