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A N I N T E R N A T I O N A L J O U R N A L

Oxidative Damage and Antioxidant Response in *Chenopodium murale* L. Exposed to Elevated Levels of Zn

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ABSTRACT

Present study was carried out to investigate the effects of different Zn concentrations on growth and some antioxidant responses in Chenopodium murale L. *The seeds were sown on the pots. 45-day plants were transferred to hydroponic solution containing 0.2 (control), 150, 300 and 600 µM Zn. The plants were harvested at 6 days after applying Zn treatments and some parameters were evaluated including plant length, fresh weight, photosynthetic pigments, malondialdehyde (MDA), ascorbate, proline and enzymatic antioxidants such as catalase (CAT), guaiacol peroxidase (GPX) and superoxide dismutase (SOD). Zn concentration showed a considerable increase in the shoot and root as the concentration of Zn increased in the medium, meanwhile the roots were characterized by higher Zn accumulation. At 150 µM Zn, the length and fresh weight did not show important changes compared to the control, but these parameters decreased at 300 and 600 µM Zn. With increasing Zn doses, the content of total chlorophyll declined, and also the content of carotenoids elevated. Excess Zn led to an increase in lipid peroxidation, free proline and ascorbate pool. Moreover, elevated Zn levels enhanced the activities of CAT, GPX and SOD.* C. murale *probably tolerates Zn concentrations up to 150 µM using the production of carotenoid, proline and ascorbate as well as enhanced activity of enzymatic antioxidants. Higher Zn treatments seem to be toxic due to a severe decline in growth.*

Key words: Ascorbate, Enzymatic antioxidant, Lipid peroxidation, Proline, Zinc

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INTRODUCTION

Zinc (Zn), as a micronutrient heavy metal, is required for normal growth and metabolism in plants. Zn is taken up by the roots mainly as Zn^{+2} free ions from soil solution [1]. Zn transporters primarily belong to ZIP (Zrt/IRT-like protein) family. Some of them are preferentially expressed and localized in the plasma membrane of root epidermal cells [2]. Zn is a structural component of the enzymes including carbonic anhydrase, alcohol dehydrogenase, superoxide dismutase (SOD) and RNA polymerase and it also serves as cofactor and activator in some enzyme reactions; it is involved in metabolism of lipids, carbohydrates and nucleic acids [3]. Moreover, integrity preservation of ribosomes and biomembranes is dependent on Zn existence in the plant [1]. Zn is also recognized to reduce oxidative stress induced by other metals [4]. Despite having a critical role in plant physiological processes, high concentrations of Zn are resulted in toxicity in plants. Toxicity symptoms and tolerance mechanisms to heavy metals vary among different plant species, even within the populations of the same species [3]. The most general symptoms of excess Zn are growth inhibition, biomass reduction, leaf chlorosis, senescence induction and photosynthesis damage. Furthermore, excess Zn can interfere with acquisition and transport of some nutrients [5,6] and leads to Mn and Fe deficiency in plant shoots [7].

In plants subjected to metal stress, an imbalance in producing and removing ROS induces oxidative stress through Haber-Weiss reaction [3,8]. ROS oxidize and destroy major biomolecules such as lipids, proteins, nucleic acids and pigments [3]. Nevertheless, the plants employ enzymatic antioxidants including superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7), together with non-enzymatic antioxidants such as ascorbate [9,10] (AsA), proline [8,11,12] to minimize harmful effects of oxidative stress and detoxify ROS caused by metal stress [8]. Some studies have been reported that excess Zn doses can induce oxidative stress, and also change antioxidant enzyme activities [8,9,10,13]. These results showed that Zn toxicity avoidance greatly depends on the activity of SOD,

CAT and guaiacol peroxidase (GPX). SOD, as first line of antioxidant defense against ROS, converts superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), which is then reduced to water and molecular oxygen by POX and CAT [14]. AsA, as a water-soluble antioxidant and redox buffer, can eliminate a variety of ROS; meanwhile contribute to reduce metal-induced oxidative stress as cofactor of ascorbate peroxidase [15]. Proline, as an organic osmolyte, is known to play principal role in osmoregulation and preventation of protein denaturation. A strong correlation between proline accumulation and antioxidant response has been reported under metal stress [16].

Chenopodium murale L. (Amaranthaceae family) broadly grows in industrial area of steel production companies, located in southeast of Ahvaz city in Iran, where smelting activities lead to release metal particles such as Zn into the environment. There is no information about the effect of heavy metal stress on physiological and biochemical responses of the plants grown in this area. This study hence was conducted to investigate the effect of different concentrations of Zn under hydroponic system on growth parameters and some of antioxidant responses in *C. murale* collected from mentioned area. These results probably will be useful to evaluate Zn tolerance by this plant species.

MATERIAL AND METHODS

Plant culture and growth conditions

Seeds of *C. murale* were collected from areas surrounding steel production industries located in southeast Ahvaz city, Khuzestan province of Iran where the activity of steel companies leads to release heavy metal particles into the environment. This area has been located in the geographical position of 31˚20´N latitude and 48˚40´E longitude. The seeds were sterilized with 20 % (v/v) sodium hypochlorite and sown on the surface of commercial soil with NPK (12.4: 4.5: 14.7) fertilizer. Irrigation was performed with tap water three times per week. The 45-day-old plants were transferred to liquid cultures with 0.1 strength modified Johnson's nutrient solution [17] containing micro and macro elements: 2.5 µM H_3BO_3, 0.2 µM $MnSO_4$. H_2O, 0.2 µM $ZnSO_4$ $7H_2O$, 0.05 µM $CuSO_4$ $5H_2O$, 0.05 µM Na_2MoO_4, 2.0 µM Fe-EDTA, 1000 µM $Ca(NO_3)_2$ $4H_2O$, 400 µM $CaSO_4$ $2H_2O$, 200 µM KH_2PO_4, 400 µM K_2SO_4. The pH of the growth medium was adjusted to 6.0 ± 0.3. In order to supply enough oxygen, nutrient solutions were continuously aerated using air pumps. After 7 days growing in the mentioned solution, the plants were transferred to new complete nutrient solutions with different concentrations of Zn $(NO_3)_2$ $6H_2O$ including 0.2 (control), 150, 300 and 600 µM for 6 days. All the steps of experiment were carried out under controlled conditions with 16 h light/8 h dark, 25 ºC/20 ºC day-night cycle. The plants were illuminated with 150 µmol photons m^{-2} s^{-1} during light period. The nutrient solutions are renewed once every 3 days. The plants were harvested 6 days after Zn treatments. Shoot and root length and fresh weight were recorded during harvesting. Following exposure to different Zn treatments, all the experiments associated to hydroponic solution were repeated three times using 10 L containers. For each replication (including different Zn concentrations), the twelve containers (35 plants per container) were prepared and used to analyze various parameters at harvest time.

Measurement of Zn concentration

At harvest time, to eliminate Zn from the root surface, the roots were completely immersed in 0.1 M EDTA- Na_2 for 10 min, and then washed three times with deionized water. After separating of roots and shoots, the plant samples were dried at 72 ºC for 48 h in an oven. Dried powders (1 g) were digested with 10 ml of 65 % HNO_3 at room temperature for overnight. The digests were heated at 85 ºC until the acid completely evaporated. Subsequently, the samples were mixed with 1 ml of 30 % H_2O_2. After filtering, the solutions were made up to a volume of 50 ml with deionized water [18]. The concentration of Zn was determined by a Flame Atomic Absorption Spectrometer instrument (GBC, Avanta model, Australia). Translocation factor (TF) and Bioconcentration factor (BF) were calculated as described by Rosén et al.[19]:

Eq. (1) TF= Zn concentration of the shoot (mg g^{-1} DW) / Zn concentration of the root (mg g^{-1} DW)
Eq. (2) BF= Zn concentration of the shoot or root (mg g^{-1} DW) / Zn concentration of the solution (mg L^{-1})

Measurement of pigment content

The content of photosynthetic pigments was assayed consistent with Lichtenthaler [20]. The 0.5 g fresh weight of leaves was homogenized in 20 ml of 80 % (v/v) acetone, and then filtered. All steps were done under ice-cold conditions and weak light. The absorbance of the samples was measured at 470, 663.2 and 646.8 nm using an UV-Vis spectrophotometer (Optision

2120UV PLUS, Korea). Total chlorophyll and carotenoid amounts were reported as mg g^{-1} FW.

Assessment of lipid peroxidation, ascorbic acid and proline

Lipid peroxidation was reported as malondialdehyde (MDA) amount in accordance with Heath and Packer [21]. Fresh weight of leaves (0.5 g) was homogenized in 5 ml of 0.1 % (w/v) trichloroacetic acid (TCA), and then centrifuged at 10000×g for 10 min. Four ml of 20 % TCA solution containing 0.5 % (w/v) thiobarbituric acid (TBA) was added to 1 ml of supernatant and incubated at 95 ºC. After 30 min, the mixture was immediately transferred to an ice bath, and again centrifuged at 10000×g for 10 min. The absorbance of supernatant was determined spectrophotometrically at 532 and 600 nm. MDA concentration was represented in µmol g^{-1} FW using the extinction coefficient equal to 155 mM^{-1} cm^{-1}.

Ascorbate measurement was performed following a modification of the method of Mukherjee and Choudhuri [22] and expressed as µg g^{-1} FW. Using 10 ml of 5 % (w/v) metaphosphoric acid, 0.5 g of fresh leaf was extracted. The homogenate was centrifuged at 10000×g for 15 min at 4 ºC. The supernatant was used to assay total (AsA + DHA) and oxidized ascorbate (dehydroascorbate; DHA) contents. To determine total ascorbate amount, 0.5 ml of 3 mM 2,6-dichloroindophenol sodium was added to 1 ml of the supernatant to oxidize ascorbic acid to DHA. To assay DHA, 1 ml of prepared supernatant was mixed with 0.5 ml of deionized water. With addition of 1 % (w/v) thiourea solution, all the tubes were left without shaking for 20 min. All the sample solutions were combined with 1 ml of 10 mM 2,4-dinitrophenylhydrazine and then placed in the water bath at 50 ºC for 1 h. The reaction was stopped by transferring the tubes to an ice bath for 30 min and subsequently, 2.5 ml of 85 % (v/v) H_2SO_4 was gradually added to the samples under ice-cold conditions. After adding 1 ml of 20 % (v/v) H_2SO_4, the amount of AsA + DHA and DHA was assayed at 520 nm using an UV-Vis spectrophotometer. Content of reduced ascorbate (AsA) was calculated by subtracting values of total ascorbate and DHA. L-ascorbic acid was used to prepare standard solutions.

Free proline assessment was done using a method described previously [23]. A 0.5 g of fresh weight was homogenized with 10 ml of 3 % (w/v) sulfosalisylic acid under cold conditions. Following centrifugation (10000×g for 10 min at 4 ºC), the supernatant was exposed to 2 ml of ninhydrin reagent and 2 ml of glacial acetic acid. All the samples were incubated in a water bath at 100 ºC for 1 h and immediately put on the ice bath to stop reaction. The samples were mixed with 4 ml of toluene, then vortexed for 30 sec. After separation of two phases, the absorbance of supernatant containing toluene and proline was measured spectrophotometerically at 520 nm. Proline amount was expressed as µmol g^{-1} FW with regard to standard solutions of proline.

Assessment of enzyme activity

A 0.2 g of leave fresh weight was homogenized with 5 ml of 100 mM potassium phosphate buffer (PSB, pH 7.0) containing 0.1 mM EDTA and 1 % (w/v) polyvinylpyrrolidone under ice-cold conditions according to Qiu et al. [24]. Homogenate was centrifuged at 15000×g for 15min at 4 ºC. The amount of soluble proteins in the supernatant was quantified as described by Bradford [25]. The supernatant was used to assay CAT, GPX and SOD activity in unit (U) mg^{-1} protein. Method of Aebi [26] was used to determine spectrophotometerically CAT activity. Reaction mixture was contained 520

µl of 50 mM PSB (pH 7.8), 250 µl of deionized water, 150 µl of 100 mM H_2O_2 and 80 µl of protein extract. The reaction was started with addition of H_2O_2 to the reaction mixture and the decrease in absorbance was monitored by the consumption of H_2O_2 at 240 nm within 2 min. One unit of CAT activity was defined as an absorbance decrease of 0.01 U min^{-1}. GPX activity was assayed according to Hemeda and Klein [27]. The reaction mixture was prepared as 1430 µl of 50 mM PSB (pH 6.0), 500 µl of 2 % (v/v) H_2O_2, 500 µl of 50 mM guaiacol and 70 µl of protein extract. Following the addition of guaiacol, the increase of absorbance in the mixture was assayed by guaiacol oxidation and tetraguaiacol formation at 470 nm for 2 min. An absorbance increase of 0.1 U min^{-1} was reported as one unit of GPX activity. The activity of SOD was determined spectrophotometerically as described by Beauchamp and Fridovich [28]. Regarding inhibition of nitrobluetetrazolium (NBT) photochemical reduction by SOD, one unit of enzyme was defined as an amount of protein required to result in 50 % inhibition of NBT reduction rate under irradiation. The reaction mixture was included 1450 µl of 50 mM PSB (pH 7.8), 300 µl of 130 mM methionine, 300 µl of 750 µM NBT, 300 µl of 0.1 mM EDTA-Na_2, 250 µl of deionized water, 300 µl of 20 µM riboflavin and 100 µl of enzyme extract. The tubes containing the reaction mixture were placed below a light source. After 15 min, the absorbance of mixture was recorded at 560 nm using a dark control.

Statistical analysis

All the experiments were performed three replicates for each treatment as completely randomized designs. The data were analyzed using one-way analysis of variance (ANOVA). The means obtained from four Zn treatments were compared with each other by Duncan's Multiple Range Test at $p < 0.05$ confidence level. Statistical analysis was carried out by the SPSS 16.0 package.

RESULTS

Uptake of Zn and effect on growth characteristics and photosynthetic pigments

According to Table 1, when the plants were supplemented with various Zn treatments, the Zn concentration in the shoots and roots showed a significant increase ($p< 0.05$) compared to control group (0.2 µM Zn). The highest Zn concentration was measured in shoots and roots grown at 600 µM Zn as 0.43 and 1.66 mg g^{-1} DW, respectively. Taking into account the values of TF, it was detected that shoot to root Zn concentration ratio was less than 1. Table 1 illustrates that the values of BF were calculated less than one in both the shoots and roots. However, the roots mainly showed higher values in BF as compared to the shoots. The length of shoot and root exhibited a significant reduction ($p< 0.05$) compared to the control at 300 and 600 µM Zn, but the plants treated with 150 µM Zn did not present considerable different for this parameter (Table 2). There was no meaningful difference between plants grown at 0.2 and 150 µM Zn in shoot and root fresh weight (Table 2). A significant decrease ($p< 0.05$) was found in total chlorophyll content of the plants grown in different Zn concentrations compared to the control and this reduction was proportional to the increase in the concentration of Zn (Fig. 1A). At concentrations of 300 and 600 Zn µM, chlorophyll contents showed a remarkable decline compared with the control. Zn treatments resulted in an important increase ($p< 0.05$) in carotenoids content as compared to the control group (Fig. 1B).

Table 1- Effect of various Zn treatments (control, 150, 300 and 600 µM) on shoot and root Zn concentration, TF and BF.

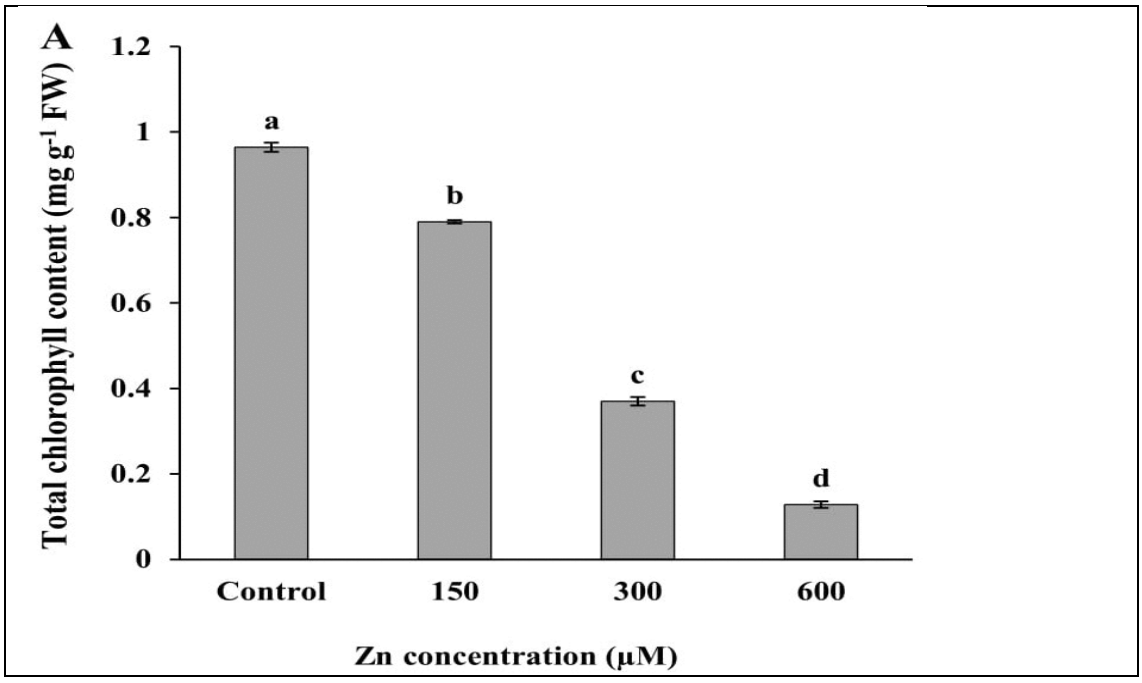
Harvest time	Zn treatment (µM)	Zn concentration (mg g^{-1} DW)		TF	BF	
		Shoot	Root		Shoot	Root
before treatment	-	0.17 ±0.01^{d}	0.21 ±0.02^{d}	0.87 ±0.11^{a}	0.10 ±0.003^{a}	0.12 ± 0.2^{d}
6 days after Treatment	(control) 0.2	0.18 ± 0.007^{d}	0.22 ± 0.02^{d}	0.82 ± 0.06^{a}	0.11 ± 0.003^{a}	0.13 ± 0.01^{d}
	150	0.33 ± 0.008^{c}	1.53 ± 0.01^{c}	0.22 ± 0.008^{d}	0.10 ± 0.007^{a}	0.46 ± 0.05^{a}
	300	0.38 ± 0.02^{b}	1.58 ± 0.01^{b}	0.24 ± 0.01^{c}	0.06 ± 0.004^{b}	0.27 ± 0.002^{b}
	600	0.43 ± 0.01^{a}	1.66 ± 0.04^{a}	0.26 ± 0.001^{b}	0.06 ± 0.001^{b}	0.24 ± 0.006^{c}

Values are expressed as mean ± SE, $N = 3$ for each treatment, means in each column fallowed by different letters are significantly different at $P < 0.05$.

Table 2- Effect of various Zn treatments (control, 150, 300 and 600 µM) on shoot and root length and fresh weight

Harvest time	Zn treatment (µM)	Length (cm)		Fresh weight (g per plant)	
		Shoot	Root	Shoot	Root
before treatment	-	4.5 ± 0.1^{a}	10.4±0.5^{a}	6.0 ± 0.02^{a}	0.16 ± 0.003^{a}
6 days after Treatment	(control) 0.2	7.3 ± 0.1^{a}	19.4 ± 0.5^{a}	7.6 ± 0.1^{a}	0.32 ± 0.02^{a}
	150	6.8 ± 0.3^{a}	18.5 ± 0. 7^{a}	7.2 ± 0.3^{a}	0.31 ± 0.01^{a}
	300	5.6 ± 0.2^{b}	14.6 ± 0.2^{b}	4.0 ± 0.1^{b}	0.15^{b}
	600	4.3 ± 0.3^{c}	14.3 ± 0.2^{b}	3.2 ± 0.1^{c}	0.12^{c}

Values are expressed as mean ± SE, $N = 3$ for each treatment, means in each column fallowed by different letters are significantly different at $P < 0.05$.



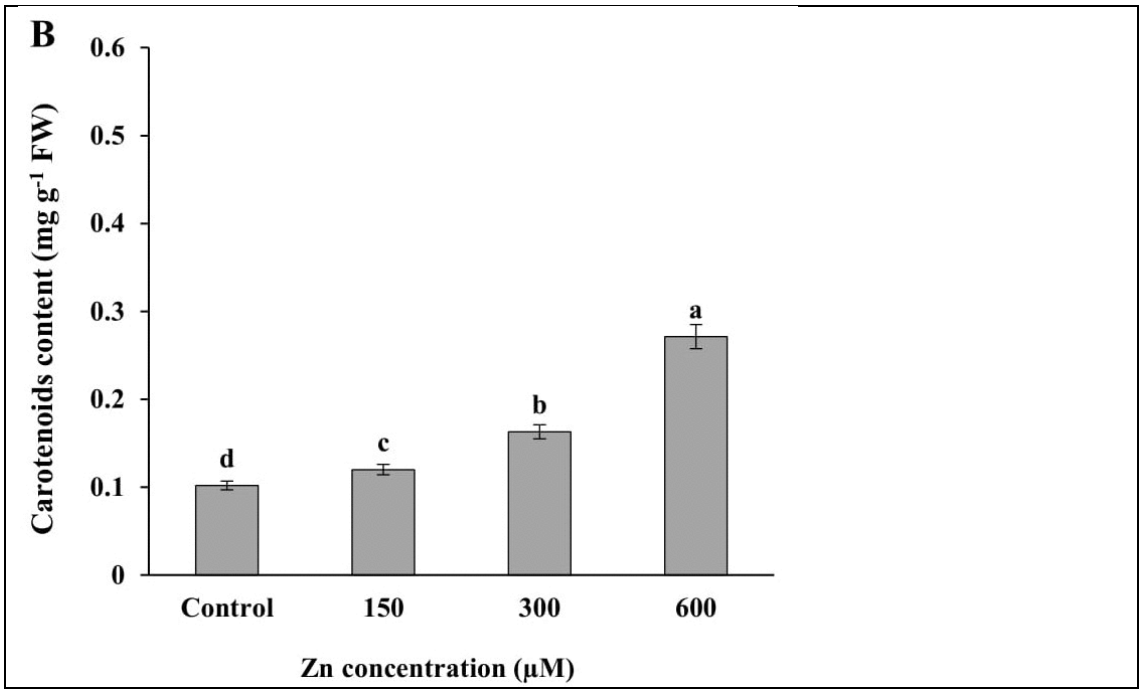
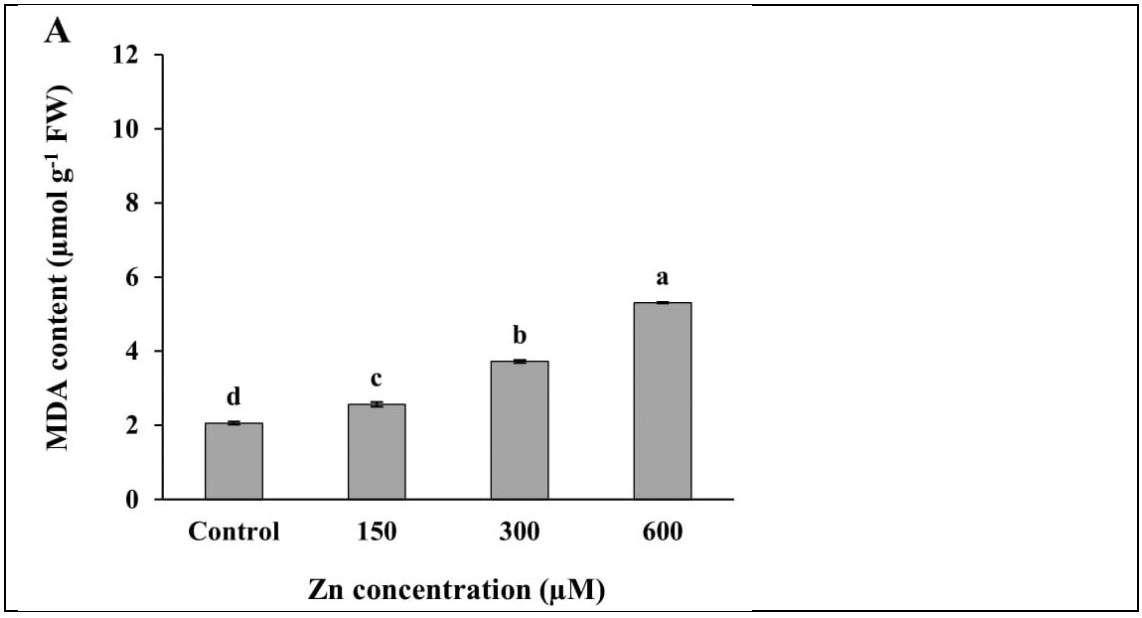
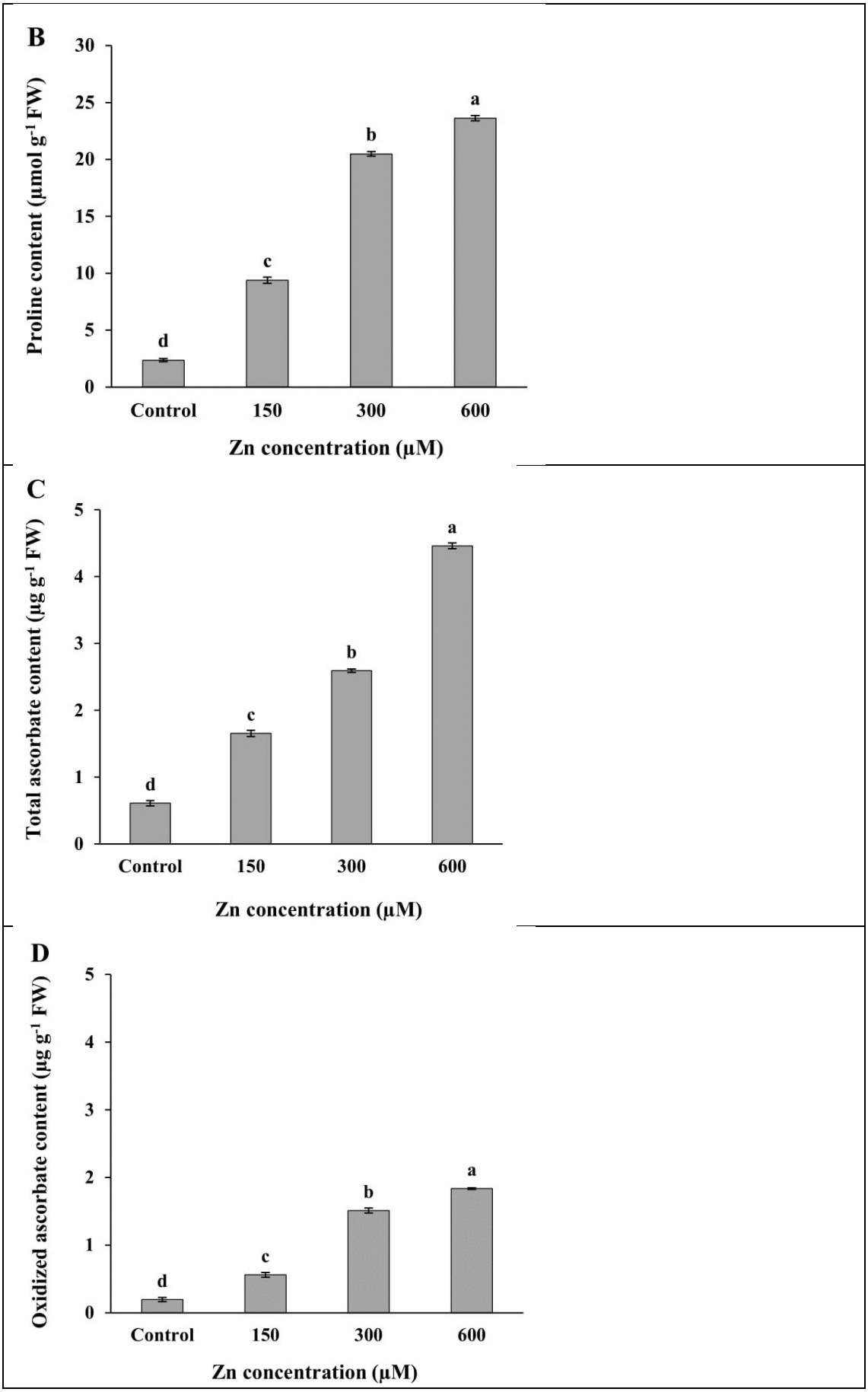


Figure 1- Effect of various Zn treatments (control, 150, 300 and 600µM) on contents of total chlorophyll (A) and carotenoid (B) after 6 days of exposure to Zn treatments. Values are mean ± SE for each treatment, $N = 3$. Different letters indicate significantly different values at $P < 0.05$ confidence level.

Effect of Zn on lipid peroxidation, proline and ascorbate

Increasing Zn supply levels were significantly effective ($p< 0.05$) on MDA content, and maximum MDA was assayed in plants treated with 600 µM Zn (Fig. 2A). The results indicated a significant increase ($p< 0.05$) in proline content in response to treatment of Zn (Fig. 2B) . The highest accumulation of proline was measured in plants grown in the culture containing 600 µM Zn. Comparing to the control group, a considerable increase ($p< 0.05$) was observed in DHA + AsA, DHA and reduced AsA for the plants which were exposed to different treatments of Zn after 6-day period (Figs. 2 C, D, E). The ascorbate pool exhibited the greatest content in the plants supplied with 600 µM Zn.




B
Proline content (µmol g-1 FW)
30
25
20
15
10
5
0
a
b
c
d
Control
150
300
600
Zn concentration (µM)
C
Total ascorbate content (µg g-1 FW)
5
4
3
2
1
0
a
b
c
d
Control
150
300
600
Zn concentration (µM)
D
Oxidized ascorbate content (µg g-1 FW)
5
4
3
2
1
0
a
b
c
d
Control
150
300
600
Zn concentration (µM)

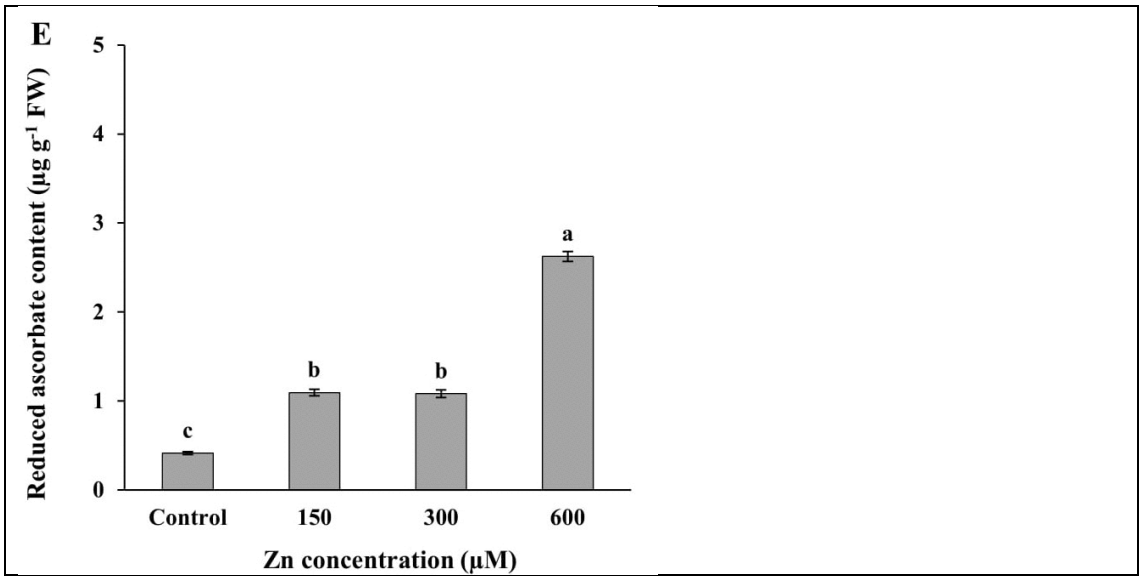
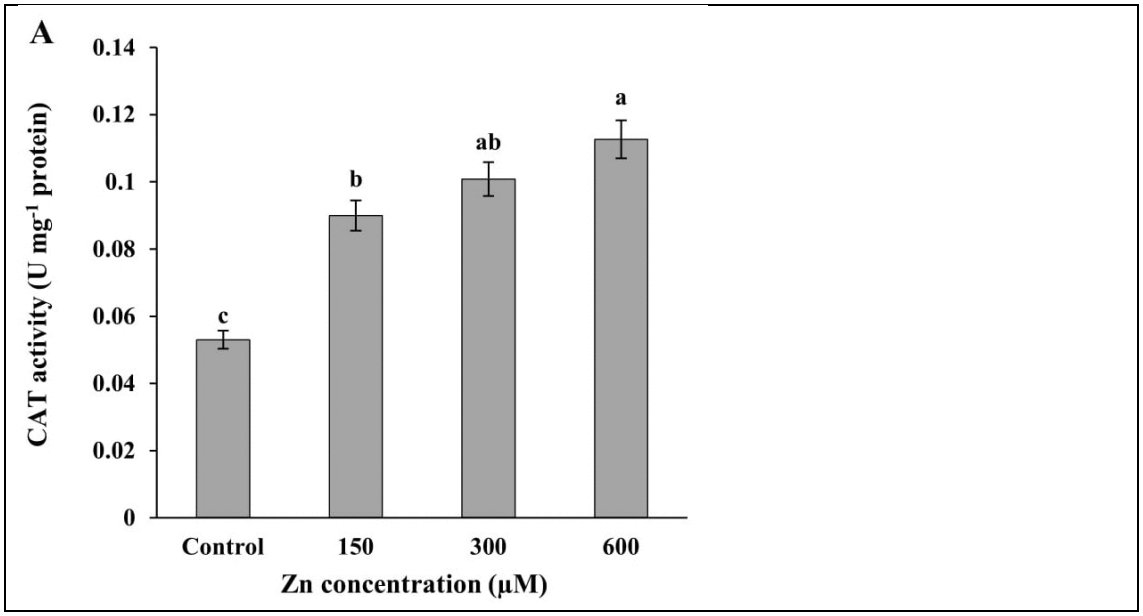


Figure 2- Effect of various Zn treatments (control, 150, 300 and 600µM) on contents of MDA (A), proline (B), total ascorbate (C), oxidized ascorbate (D) and reduced ascorbate (E) after 6 days of exposure to Zn treatments. Values are mean ± SE for each treatment, $N = 3$. Different letters indicate significantly different values at $P < 0.05$ confidence level.

Effect of Zn activity of antioxidative enzymes

The effect of various concentrations of Zn on enzyme activity is presented in Figure 3. A significant increase ($p< 0.05$) depended on Zn concentration in activity of enzymatic antioxidants including CAT, GPX and SOD was observed comparing to the control group during the experiment period. As shown in Figure 3A, the CAT activity did not show critical differences among the plants treated with 150, 300 and 600 µM Zn, but it increased in comparison with the control. There were significant differences ($p< 0.05$) among the plants grown at Zn concentrations higher than the control concentration in both GPX and SOD activities (Figs. 3 B, C).



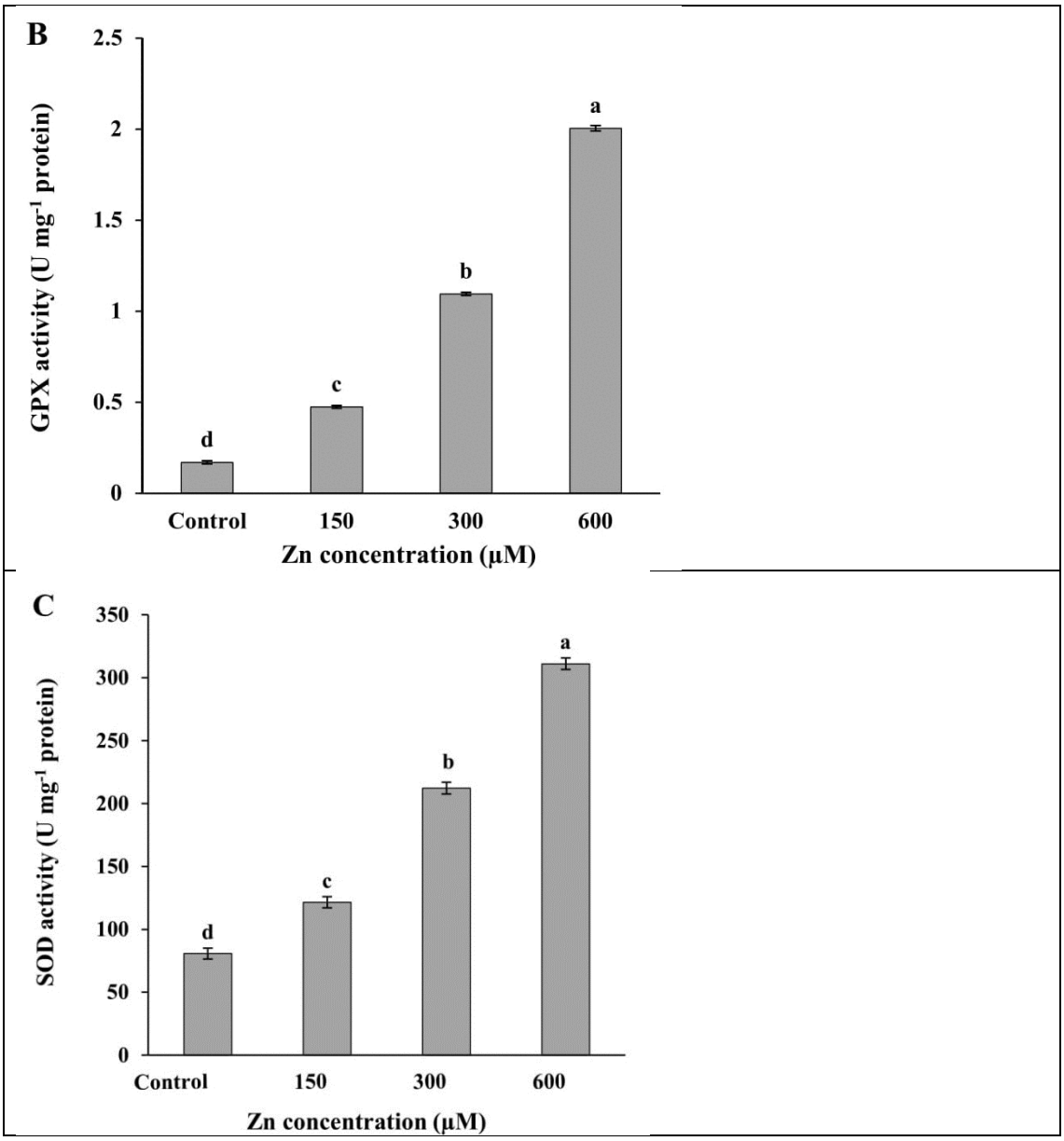


Figure 3-. Effect of various Zn treatments (control, 150, 300 and 600 µM) on activity of CAT (A), GPX (B) and SOD (C) after 6 days of exposure to Zn treatments. Values are mean ± SE for each treatment, N = 3. Different letters indicate significantly different values at $P < 0.05$ confidence level.

DISCUSSION

In this study, Zn uptake by both shoots and roots showed an increase with increasing Zn concentrations in the growth medium. However, given organ Zn concentrations, and also TF and BF amounts, the roots exhibited the ability of higher Zn accumulation when compared with the aerial parts (Table 1). It has been pointed out that higher metal accumulation in roots is used as a tolerance strategy to prevent translocation of excess metal levels to photosynthetic organs and protect them against toxicity induced by metal accumulation [29]. A raise in root BF (compared to shoot BF) with increasing Zn concentration in the solution, indicates that most Zn absorbed by *C. murale* is concentrated in the roots and a small fraction transported to the shoots to diminish Zn stress.

Toxicity caused by the concentrations higher than 150 µM Zn significantly reduced growth of the plants (Table 2). The growth and biomass

maintenance is one of the most important properties in the plants to tolerate metal stress [10]. Some plants grown under Zn stress represented a strong decrease in organ elongation and biomass production [7,9,5,30,31]. Toxic Zn doses are known to exert the negative effects on the growth of plant by way of injury in nutrient elements availability [30,31], disturbance in water balance and creating oxidative stress [10]; moreover, excess Zn prevents metabolic processes related to plant growth [32]. In this study, with increasing in Zn accumulation, the growth of shoots and roots presented a remarkable decline. Therefore, these results indicate that higher uptake can reduce the growth of *C. murale* through Zn-induced toxicity, and also lipid peroxidation enhancement (Fig. 2A) and oxidative damage initiated by these doses may be some important reasons to decline growth and biomass production of *C. murale*.

In the present study, higher Zn concentrations considerably reduced total chlorophyll content. Nevertheless, the content of carotenoids showed an improving trend (Fig. 1). The decline in chlorophyll contents has been reported as one of the most general symptoms in some plant species exposed with toxic Zn levels [4,8,9,30,33]. Decrease in chlorophyll contents of the plants exposed to excess Zn concentrations may be due to either the decrease in chlorophyll synthesis or increase in its decomposition [34]. Li et al. [8] have suggested that higher Zn doses probably destroy chloroplast membranes and induce chlorophyll hydrolysis. Chloroplast is one of important organelles to create ROS such as $O^{\cdot-}_2$, H_2O_2 and $^{\cdot}OH$ (hydroxyl radicals). Elevated Zn can inhibit function of photosynthetic electron transport chain and thus induce ROS generation and oxidative stress [35]. Consequently, enhanced lipid peroxidation under higher Zn levels, can support the theory of chloroplast destruction and lead to a decline in chlorophyll content in *C. murale*. In *Myracrodruon urundeuva* under toxic Zn doses, enhanced carotenoids content has been attributed to their protective role for photosynthetic apparatus against ROS accumulation and oxidative stress [10]. ROS created by various stresses is suggested to support carotenoid biosynthesis by activating enzymes and genes involved in carotenogenesis [36]. Carotenoids display a pivotal role to scavenge $^1O_2$ (singlet oxygen) produced by other ROS types [37]. Hence, our study proposes that carotenoids accumulation is associated to their key function in protecting the plant cells against Zn- induced oxidative stress.

Following exposure to different Zn treatments, levels of MDA, free proline and ascorbate pool showed an increased trend with increasing Zn concentrations (Fig. 2) . It has previously been reported that toxic levels of Zn stimulate ROS production and lipid peroxidation (for example [10,12,15]). Oxidative stress mediated by non-redox metals such as Zn has been ascribed to a rapid generation of H_2O_2 and $O_2^{\cdot-}$ in tobacco [38]. Indeed lipid peroxidation is one of the first consequences of oxidative stress. Intensification of lipid peroxidation due to excess Zn in plans has been attributed to in increasing activity of membrane-bound lipoxygenase, which leads unsaturated fatty acids oxidation, oxidative stress induction and finally MDA generation [15,32]. Degree of tolerance to the oxidative stress induced by metals is highly dependent on enzymatic and non-enzymatic antioxidants function, plant species and metal toxicity [39]. Some studies have illustrated that free proline content is enhanced under Zn stress conditions [8,12,40]. Metal-induced proline accumulation is supposed to be due to its ability to scavenge $^{\cdot}OH$ and $^1O_2$, also proline is able to act as an osmolyte and metal chelator in these conditions [16]. In the present study, a simultaneous increase in lipid peroxidation and proline content in *C. murale* could illustrate a possible role for proline in reducing Zn-induced oxidative stress. At higher

concentrations, Zn has led to an increase in DHA contents in *Brassica napus* and *Hydrilla verticillata* [9,15], suggesting that redox status of the cell changes to more oxidized forms of ascorbate. An increase in total ascorbate and DHA contents has been observed as doses of Zn increased in *M. urundeuva* [10]. Ascorbate, as the most abundant non-enzymatic antioxidant, exhibits a critical role in ascorbate-glutathione cycle to diminish H_2O_2 damage in chloroplasts [41]. In addition, ascorbate directly acts to scavenge other types of ROS [32]. In present study, increased ascorbate pool probably implies the active participation of this non-enzymatic antioxidant to eliminate ROS and reduce Zn-induced oxidative stress.

Under metal stress conditions, the function of antioxidant enzymes is exerted through the elimination of ROS [14]. Our study clearly indicates that Zn-induced lipid peroxidation increasing SOD, CAT and GPX activities and can confirm generation of $O^{\cdot-}_2$ and H_2O_2. Moreover, increased the activity of these enzymes may be due to the maintenance of balance in the contents of $O^{\cdot-}_2$ and H_2O_2 under Zn stress. Toxic Zn doses is known to alter differently the activity of antioxidant enzymes such as SOD, CAT and POX in *Thlaspi caerulescens* [13], *Verbascum thapsus* [42], *Brassica napus* [9], *Eruca sativa* [43] and *Triticum aestivum* [8]. It seems that performance of antioxidant network against metal stress is particularly determined by plant species, plant organs, metal concentrations, growth conditions and exposure times. The conflicting observations in the activity of enzymatic antioxidants may be due to expression differences in genes coding these enzymes under toxic Zn concentrations [44]. Zn, as a cofactor, is required for structural and functional stability of Cu/Zn-SOD, which is the most important isoenzyme to remove $O^{\cdot-}_2$ in plant cells [45], it hence has been suggested that excess Zn may lead to increase activity of SOD [10]. CAT is mainly active in peroxisomes and mitochondria, while POX acts in cytoplasm, membrane and cell wall. Both enzymes play a significant role in detoxification of H_2O_2. To detect the total POX activity, guaiacol is usually used as a non-specific electron donor to H_2O_2, in such cases; peroxidase is referred as guaiacol peroxidase [9]. High Zn concentrations were found to upregulate POX genes and increase activity of isoforms involved in wall lignifications to avoid oxidative damage [44]. Based on the results of this study, both CAT and GPX activity increased in response to different Zn levels as compared to the control. However, at the concentrations more than 150 µM Zn, GPX activity presented a dose dependent increasing trend, whereas there was no remarkable difference in CAT activity among the plants grown in these treatments. Hence, it seems that GPX illustrates a more important role as compared to CAT to remove H_2O_2 created by toxic Zn levels in *C. murale.*

CONCLUSIONS

Taken together, based on these results, increased Zn concentration in the medium raised Zn uptake and accumulation in both shoots and roots. Nevertheless, the roots had higher capability to accumulate Zn compared to the aboveground parts. The *C. murale*, the population collected from industrial region surrounding steel production companies in Ahvaz, supposes to be tolerant to Zn concentrations up to 150 µM due to maintenance of the growth and biomass. At higher Zn concentrations applied in the present study, the enzymatic and non-enzymatic antioxidants exhibited an important increase. However, these concentrations significantly decreased the plant growth compared to the control. This study; therefore, supports the idea that excess Zn doses induce oxidative stress and antioxidant defense in *C. murale*. However, improved function of antioxidant systems along with

increasing plant Zn concentration would indicate that *C. murale*, at least up to 150 µM, effectively increases antioxidative activities to resist against Zn-induced toxicity. At higher concentrations, concerning significant reduction of growth, it is assumed that enhanced antioxidants system is preferentially a consequence of Zn stress, and hence is not probably a signal of Zn tolerance.

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