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IMPACT OF EXCESS ZINC ON PHOTOSYNTHETIC PIGMENTS AND ANTIOXIDATIVE ENZYME ACTIVITIES OF RUDERAL *VERBASCUM OLYMPICUM* BOISS.

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ABSTRACT

Verbascum olympicum Boiss. is dominated in disturbed areas of Uludağ Mountain/Turkey. It is a special species that is subject to phytoremediation due to its heavy metal accumulation, high biomass production and its ability to live in degraded areas. But, it is not known what its photosynthetic and antioxidative responses to high Zn concentrations that increased with anthropogenic activities such as mining, sewage sludge. Thus, in the present study we investigated photosynthetic pigments (chlorophyll *a* and *b*) and antioxidative enzymatic system of ruderal *Verbascum olympicum* grown in three different Zn levels (50, 250 and 500 µM) as zinc sulfate during 1, 3 and 10 days. The activity of antioxidative enzymes [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX)] and photosynthetic pigment contents (chlorophyll *a* and *b*) was determined in the 8-week-old seedlings. Also, lipid peroxidation was measured as the amount of malondialdehyde (MDA) and, the cell membrane integrity and permeability was determined. Results were subjected to two-way ANOVA according to different Zn concentration treatments and exposed periods. It was determined an increase tendency up to 200 µM Zn treatment in antioxidative enzyme activities depending on increasing Zn concentrations and duration of exposure. This situation indicates that *Verbascum olympicum* has a strong antioxidative defense system for scavenging Zn-induced oxidative stress. But 500 µM Zn treatment had a destructive effect in photosynthetic pigment content and antioxidative enzymatic activities of *V. olympicum*. These results contribute to the understanding of survival success of *Verbascum olympicum* in degraded areas.

Key words: Antioxidative enzyme activity, chlorophyll content, zinc, *Verbascum olympicum*.

1. INTRODUCTION

Zinc (Zn) is so special micronutrient that involved in a lot of physiological and biochemical processes from plasma membrane function, chlorophyll biosynthesis, to oxidative stress tolerance, regulation of

DNA transcription and protein-protein interactions. But in the high concentrations, Zn begin to effect negatively because of binding of zinc to various functional groups of ligands containing sulfur and oxygen. In the high Zn concentrations, it causes alterations such as reduction of plant root length, chlorosis, inhibition of photosynthesis (1). Especially, it decreases the level of plastoquinone in the electron transport chain during the process of photosynthesis. It also causes irreversible damage to chlorophyll biosynthesis (2). Moreover, uptake of micronutrient elements such as Mg, Mn and Fe are prevented (3). For example; in *Phaseolus vulgaris* plants treated with high Zn concentrations, zinc has been reported to be effective on photosynthesis by replacing of magnesium in Photosystem II (4). There were also indicated by Ambler et al. (5) that high Zn concentrations create Fe deficiency and low Zn concentrations cause Fe accumulation. This situation can create toxic reactive oxygen radicals (ROS) via Fenton and Haber-Wiess reactions or electron transport chain (ETS) in mitochondria and chloroplast (6,7)

ROS give rise to the production of malondialdehyde (MDA), as well as starting the peroxidative chain reaction involving membrane lipids (8). Besides, they can cause damage to protein and even DNA or RNA (9,10). Therefore, plants have complex ROS scavenging mechanisms including antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) or low molecular weight antioxidants like ascorbic acid, glutathione, cysteine, α-tocopherol, phenolic compounds (7). The enzymatic action of SOD is the first step for scavenging ROS such as superoxide ($O_2^{\cdot -}$) and ultimately generates hydrogen peroxide (H_2O_2). Due to be toxic in high H_2O_2 concentrations, it turns to H_2O and O_2 by CAT localized in peroxisomes. APX catalyzes the reduction of H_2O_2 using ascorbate as an electron donor in chloroplast and mitochondria (11).

Verbascum olympicum is native to the local flora of disrupted areas such as the roadsides, rubber hips, developed building areas and mining areas in Uludağ (Bursa-Turkey). At the same time, *Verbascum olympicum* has some functional properties like high nitrate assimilation capacity, heavy metal accumulation (12) and high organic matter production (13). If this species becomes a dominant plant in disrupted areas, it should have different metabolic functions against heavy metal pollution. In this study, we aimed to determine the impact of different Zn concentrations (0, 50, 200, 500 µM) during 1, 3 and 10 days on the photosynthetic pigments and antioxidative enzymatic system of *V. olympicum.* We analyzed the chlorophyll contents (chlorophyll *a* and *b*), the cell membrane integrity and permeability, the level of lipid peroxidation (MDA content) and the activity of antioxidative enzymes (SOD, CAT and APX) in both leaves and roots of the *V. olympicum* seedlings growing under hydroponic conditions.

2. MATERIALS AND METHODS

2.1. Plant Culture and Experimental Design

Seeds, collected from Uludağ Mountain (1850-1900 m elevation), were stored in paper bags in room conditions. Sterilized seeds with 5% sodium hypochlorite for 5 min were planted in Petri dishes with water-moistened filter paper. Petri dishes were enclosed with aluminum foils for germination in dark conditions and incubated at 20°C for 48h. For the development of cotyledons in germinating seeds, petri dishes were wrapped with stretch film and placed in a growth chamber (Heraeus Vötsch HPS500, 15°C/25°C day/night temperature, 16h photoperiod). On the 10 days after sowing, seedlings with two cotyledons were transferred to polyvinyl chloride plates floating on 10% strength of Hoagland nutrient solution (14). This solution was renewed every other day and its concentration was increased by 10% every week. Plants were grown in these conditions for eight weeks. Then uniform plants with 8-leaves were selected for Zn treatments in form of $ZnSO_4$. 0 (control), 50, 200 and 500 µM Zn was applied to plants in 80% strength of Hoagland nutrient solution. Five plants for per treatment were used and three replications for per plant were done. Plants were harvested at 1st, 3rd and 10th day of treatment. Plant

samples were washed thoroughly with de-ionized water and separated to roots and leaves portions. Fresh plant materials were used for determination of growth parameters such as the water content, biomass production and the cell membrane injury and permeability; the rest were stored in -80°C after freezing in liquid nitrogen for others.

2.2. Determination of Photosynthetic Pigment Contents

The chlorophyll content (chlorophyll *a* and *b*) was determined using the method of Arnon (15). Fresh leaves (0.05 g fresh weight; FW) were homogenized with 10 mL of 80% acetone, and then filtered. The absorbance of samples was measured at 645 and 663 nm (Novaspec II, LKB Biochrom).

2.3. The Cell Membrane Permeability and Electrolyte Leakage Assay

Estimation of the cell membrane permeability was determined with the electrolyte leakage as described by Masood et al. (16). Fresh leaf (0.5 g) and root samples (0.25 g) were cut into pieces and immersed in 20 mL distilled de-ionized water. They were incubated in a water bath at 32ºC for 2 h and the initial electrical conductivity of the medium (EC_1) was measured. Then, the samples were autoclaved at 121ºC for 20 min to release all electrolytes. After cooled to 25ºC, the final electrical conductivity (EC_2) was measured. Electrolyte leakage was calculated as the percentage of the initial value (EC_1) over the final value (EC_2).

$$\text{The cell membrane permeability (\%): } (EC_1/EC_2)*100$$

Also, the cell membrane injury was expressed according to formula in above as a percentage (17):

$$\text{The cell membrane injury (\%): } 1-(1- EC_1/EC_2)/(1- EC_1^*/EC_2^*)$$

where, E^* is the electrical conductivity of the control sample.

2.4. Determination of Lipid Peroxidation

Lipid peroxidation was measured as the amount of MDA determined by thiobarbituric acid (TBA) reaction and was expressed as nmol/g fresh weight. MDA content was determined as proposed by Heath and Packer (18), with some modifications. 0.1 g fresh leaves material was homogenized in 0.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 g for 10 min. Then, the reaction mixture containing 0.5 mL of the supernatant and 1.5 mL of the mixture of 20% TCA and 0.5% TBA was put in new tubes. After incubation at 95 °C for 30 min, the tubes were cooled with ice and centrifuged at 15 000 g, 4°C for 5 min. The absorbance of the mixture was read at 532 and 600 nm (Novaspec II, LKB Biochrom). The MDA concentration was calculated using extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.5. The Determination of the Antioxidative Enzymatic System

1 g fresh plant material was homogenized with 3 mL of buffer solution [Buffer solution; 50 mM Na-phosphate buffer (pH 7.8), 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone (PVP)] in an ice bath. Homogenized materials were centrifuged at 14.000 g for 40 min at 4°C. The supernatants were transferred to Eppendorf tubes for the determination of the enzymatic activities. Only for the APX activity assay, 2 mM asborbate was added to the extraction buffer solution described above. The SOD activity (EC 1.15.1.1) was determined by the method of Beuchamp and Fridovich (19). This method is based on the inhibition of the nitroblue-tetrazolium at 560 nm (Novaspec II LKB Biochrom). SOD assay kit (SOD S7446, Sigma-Aldrich, USA), derived from bovine erythrocyte was used in the preparation of standards. After the calculation of % inhibition, the activity of enzyme was determined according to the linear equation which is obtained from the curve and expressed as units per mg protein.

The CAT activity (EC 1.11.1.6) was assayed as described in Lester et al. (20) with some modification. 0.1 mL of the enzyme extract was added to 20 mM sodium phosphate buffer (pH 6.8) and 15 mM H_2O_2. The change in absorbance was measured at 240 nm for 3 minute (Shimadzu UV-2100). The activity of enzyme was expressed as units per mg protein. The APX activity (EC 1.11.1.11) was determined according to the method described by Lester et al. (20). This enzyme activity was stated from the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture was comprised from 50 mM potassium phosphate (pH 6.6), 0.25 mM ascorbate and 1 mM H_2O_2 (3% H_2O_2) and 1 mL of the enzyme extract. The change in absorbance was measured at 290 nm for 3 minute (Shimadzu UV-2100). The activity of enzyme was expressed as units per mg protein.

2.6. Statistical Analysis

Results were expressed in figures and tables as mean values ± SD. The experiments were set up in a completely randomized design. According to different Zn concentration treatments and exposed periods, data were subjected to two-way ANOVA using the SPSS 16.0 for Windows (SPSS Inc. 2007). The statistical significance of the results was at 0.05 levels.

3. RESULTS and DISCUSSION

The chlorophyll *a* and *b* content determined in the leaves of the *V. olympicum* seedlings exposed to Zn at 50, 200 and 500 µM concentrations for 1,3 and 10 days was given in Figure 1. Accordingly, a significant difference was found in the mean chlorophyll *a* and *b* content depending on the durations of exposure and Zn concentrations ($P < 0.05$). It was determined that chlorophyll *a* and *b* content of *V. olympicum* seedlings decreased depending on increasing Zn concentrations and durations of exposure. The lowest chlorophyll *a* and *b* content obtained was determined in samples exposed to 500 µM Zn stress for 10 days. This value is 0.51 ± 0.03 mg / g FW for chlorophyll *a* and 0.18 ± 0.02 mg / g FW for chlorophyll *b*. High Zn concentrations might have led to the Fe deficiency in *V. olympicum* and this may have damaged the process of photosynthesis and chloroplasts (2, 21). At the same time, it can be thought that zinc has caused problems in the process of photosynthesis due to it inhibits to the intake of nutrients, since it has a similar ion charge with elements such as Mn, Mg and Fe contained in photosynthetic devices (22).

In our study, it was observed that the content of MDA increased with Zn treatments in *V. olympicum* seedlings ($p<0.05$, Figure 2). Especially in 10day treatment, increasing MDA level is remarkable (Figure 2). This indicates that oxidative stress occurs in *V. olympicum*. Because the level of oxidative stress occurring in the plant can be understood from the content of MDA and the primary effect of the formed free radicals is the cell membranes (23). Therefore, cell membrane damage and ion leakage values was measured in *V. olympicum* exposed to different Zn concentrations for 1,3 and 10 days (Table 2). It was seen in our results that cell membrane damage and ion leakage values also increased depending on Zn concentrations and durations of exposure ($p<0.05$). It has been reported by many researchers that oxidative stress occurs in plants and cell membrane integrity is impaired due to different Zn treatments (6, 24, 25, 26).

SOD activity in the leaves and roots of *V. olympicum* seedlings exposed to different Zn concentrations for 1,3 and 10 days was shown in Figure 3. SOD activity was stimulated by Zn treatments in both the roots and leaves. This stimulation was up to 200 µM Zn concentrations proportional to the increasing Zn concentrations and durations of exposure ($p<0.05$). The highest SOD activity in the roots and leaves was determined in the seedlings exposed to 200 µM Zn for ten days. This indicates that *V. olympicum* seedlings have a strong defense system that can resist to 200 µM Zn concentrations, but 500 µM Zn treatment inhibits the antioxidative defense system. Because the decrease in SOD activity is an evidence

that the enzyme is structurally degraded. The most common isoform of SOD in plants is Cu/Zn-SOD. Therefore, the changes in Zn concentrations is directly effective on the SOD activity (6).

The CAT Activity in the leaves and roots of *V. olympicum*, shown in Figure 4, increased on all the sampling days at the 50µM and 200µM Zn treatments ($p<0.05$). The lowest CAT Activity in both the roots and leaves was obtained in the seedlings exposed to 500µM Zn on the 10th day (Figure 4). The other Zn treatments, except 500µM Zn, led to remarkable elevation of the APX activity in the leaves and roots. The APX activity at 50 and 200µM Zn treatments raised with increasing Zn concentration during the ten days ($p<0.05$, Figure 5). The highest APX activity was found in leaves and roots of the seedlings exposed to 200µM Zn for 10^{th} days. The APX activity at 500µM Zn treatment decreased parallel with durations of exposure in the leaves. The results obtained in CAT and APX activities of *V. olympicum* seedlings are similar to SOD activity. Both enzyme activities decreased at 500 µM Zn concentration depending on the duration of exposure. Luo et al. (27) examined the effect of 0, 0.25, 0.5, 1, 2 and 3 mM Zn concentrations in a study with *Jatropha curcas* seedlings and stated that there was an increase in CAT activity up to 2 mM Zn concentration.

4. CONCLUSION

Enzymatic studies about determine the antioxidative defense system in plants against heavy metal treatments in high concentrations are very popular and the relationship between these enzymatic studies and the tolerance mechanisms developed by plants against heavy metals is questioned (28, 29, 30). In this study, it was determined the changes in photosynthetic pigments and antioxidative defense system of *Verbascum olympicum* which has been investigated for heavy metal deposition including Zn (31). This indicates that *V. olympicum* seedlings have a strong defense system that can resist to 200 µM Zn concentrations, but 500 µM Zn treatment inhibits the photosynthetic pigments and antioxidative defense system. These results provide basic data on the mechanisms which *Verbascum olympicum* has in cope with applied heavy metal and the impact of these mechanisms on developing in disrupted areas of Uludağ Mountain and performing their role in the secondary succession process.

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Table 1. Electrolyte leakage and Cell membrane injury in the roots and leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at $p<0.05$ compared to the control. Root Electrolyte leakage, FConcentration$(3,48) = 757.478$, $p = .000$, FDuration$(2,48) = 121.382$, $p = .000$, FConcentration x Duration$(6,48) = 22.809$, $p = .000$. Leaf Electrolyte leakage, FConcentration$(3,48) = 1569.04$, $p = .000$, FDuration$(2,48) = 205.84$, $p = .000$, FConcentration x Duration$(6,48) = 34.33$, $p = .000$. Root Cell membrane injury, FConcentration$(3,48) = 1159.94$, $p = .000$, FDuration$(2,48) = 185.97$, $p = .000$, FConcentration x Duration$(6,48) = 35.02$, $p = .000$. Leaf Cell membrane injury, FConcentration$(3,48) = 1949.64$, $p = .000$, FDuration$(2,48) = 64.84$, $p = .000$, FConcentration x Duration$(6,48) = 20.24$, $p = .000$.

		Electrolyte leakage (%)			Cell Membrane Injury (%)		
	Concentrations	**1.day**	**3.day**	**10.day**	**1.day**	**3.day**	**10.day**
Leaves	**Control**	33.6 ± 1.38	33.3 ± 2.44	33.4 ± 1.03	0	0	0
	50 µM Zn	42.3 ± 1.36	43.6 ± 1.30	62.5 ± 0.87	13.2±2.05	15.2±1.96	17.5±2.99
	200 µM Zn	52.4 ± 1.37	55.7 ± 1.53	70.6 ± 1.02	28.5±2.06	33.4±2.30	40.3±2.29
	500 µM Zn	76.9 ± 1.85	87.6 ± 3.86	91.8 ± 2.57	65.2±2.78	81.4±5.81	92.7±3.94
Roots	**Control**	53.5 ± 1.65	53.4 ± 2.15	53.5 ± 2.16	0	0	0
	50 µM Zn	63.5 ± 0.46	68.1 ± 0.72	70.6 ± 1.12	21.5 ± 0.99	31.4 ± 1.54	61.7 ± 2.41
	200 µM Zn	71.1 ± 1.67	76.6 ± 2.66	81.9 ± 0.70	37.9 ± 3.59	49.6 ± 5.72	74.2 ±1.51
	500 µM Zn	74.7 ± 2.30	86.1 ± 1.51	94.1 ± 1.65	45.7 ± 4.94	70.2 ± 3.24	87.3 ± 3.55

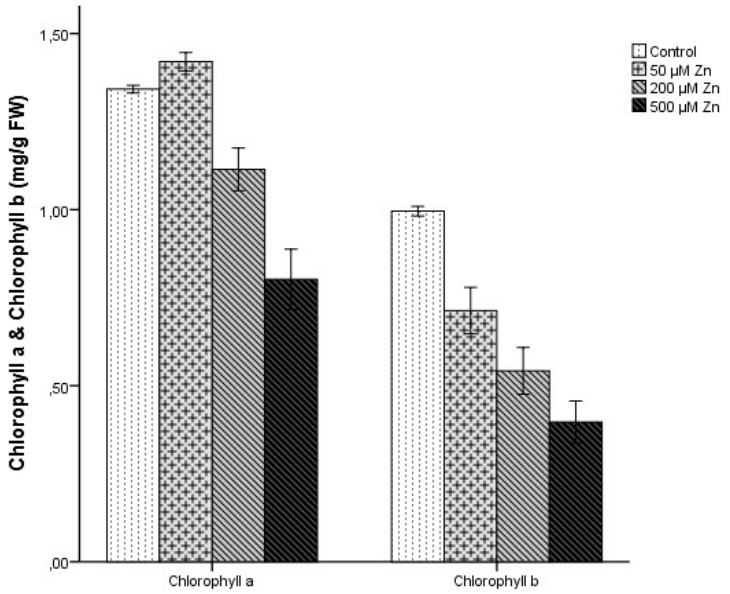

Figure 1. Chlorophyll *a* and *b* content in the leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at $p<0.05$ compared to the control. Chlorophyll *a* content, FConcentration$(3,48) = 592.14$, $p = .000$, FDuration$(2,48) = 103.31$, $p = .000$, FConcentration x Duration$(6,48) = 82.49$, $p = .000$. Chlorophyll *b* content, FConcentration$(3,48) = 286.81$, $p = .000$, FDuration$(2,48) = 174.81$, $p = .000$, FConcentration x Duration$(6,48) = 18.27$, $p = .000$.

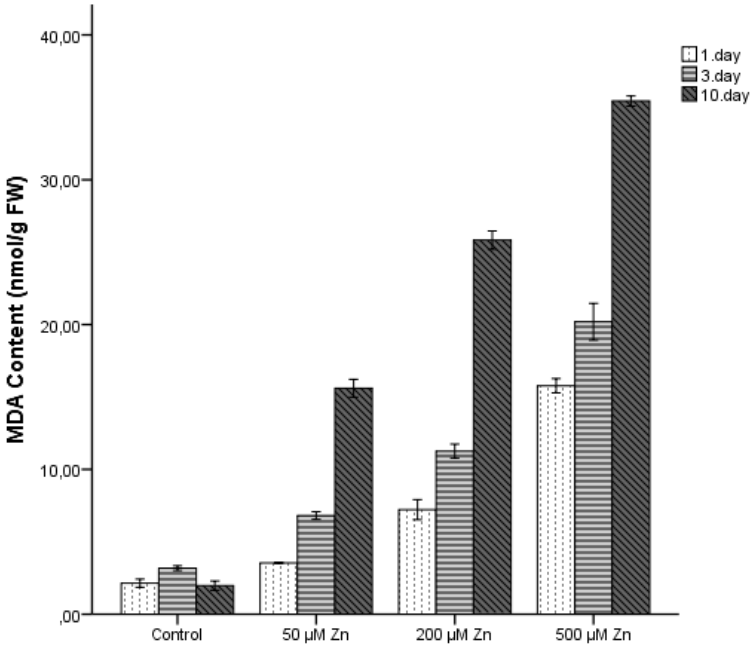

Figure 2. MDA content in the leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at $p<0.05$ compared to the control. FConcentration$(3,48) = 262.26$, $p = .000$, FDuration$(2,48) = 193.71$, $p = .000$, FConcentration x Duration$(6,48) = 28.907$, $p = .000$.

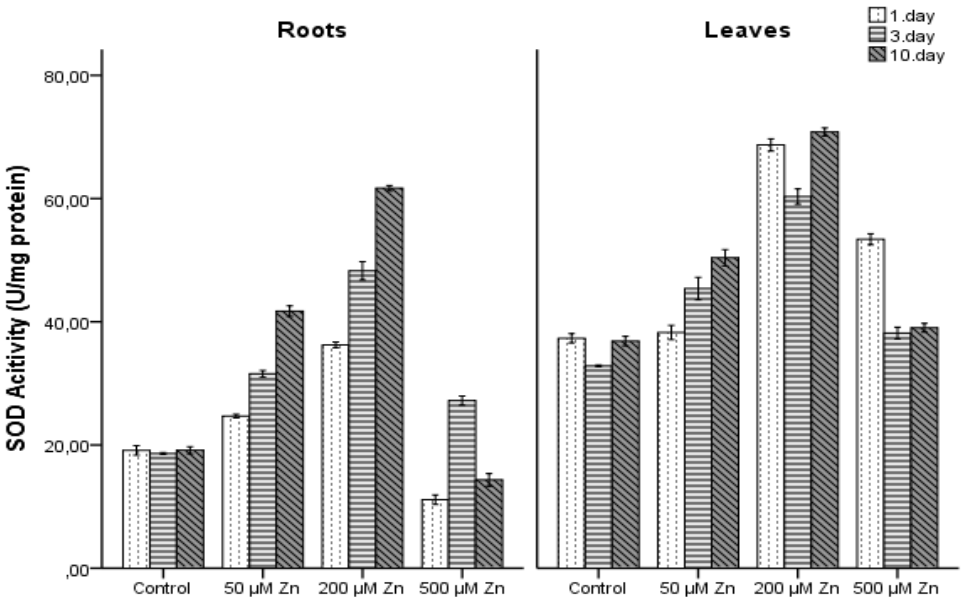

Figure 3. SOD activity in the roots and leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at $p<0.05$ compared to the control. Root SOD Activity, FConcentration$(3,48)$ = 440.986, p = .000, FDuration$(2,48)$ = 85.90, p = .000, FConcentration x Duration$(6,48)$ = 42.16, p = .000. Leaf SOD Activity, FConcentration$(3,48)$ = 210.68, p = .000, FDuration$(2,48)$ = 5.235, p = .009, FConcentration x Duration$(6,48)$ = 14.203, p = .000.

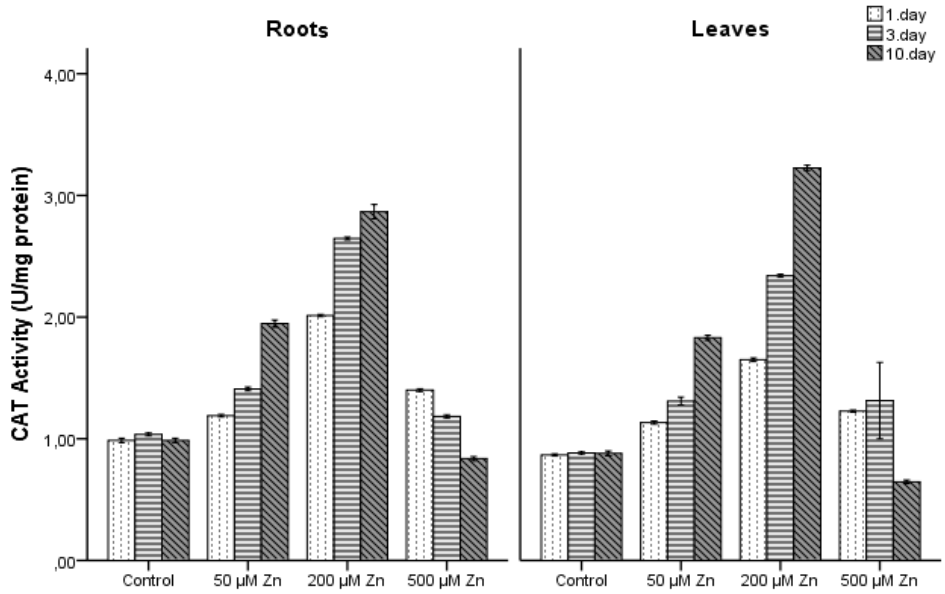

Figure 4. CAT activity in the roots and leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at $p<0.05$ compared to the control. Root CAT Activity, FConcentration$(3,48)$ = 1286.70, p = .000, FDuration$(2,48)$ = 82.12, p = .000,

*F*Concentration x Duration*(*6,48*)* = 121.14, *p* = .000. Leaf CAT Activity, *F*Concentration*(*3,48*)* = 3672.81, *p* = .000, *F*Duration*(*2,48*)* = 475.53, *p* = .000, *F*Concentration x Duration*(*6,48*)* = 550.42, *p* = .000.

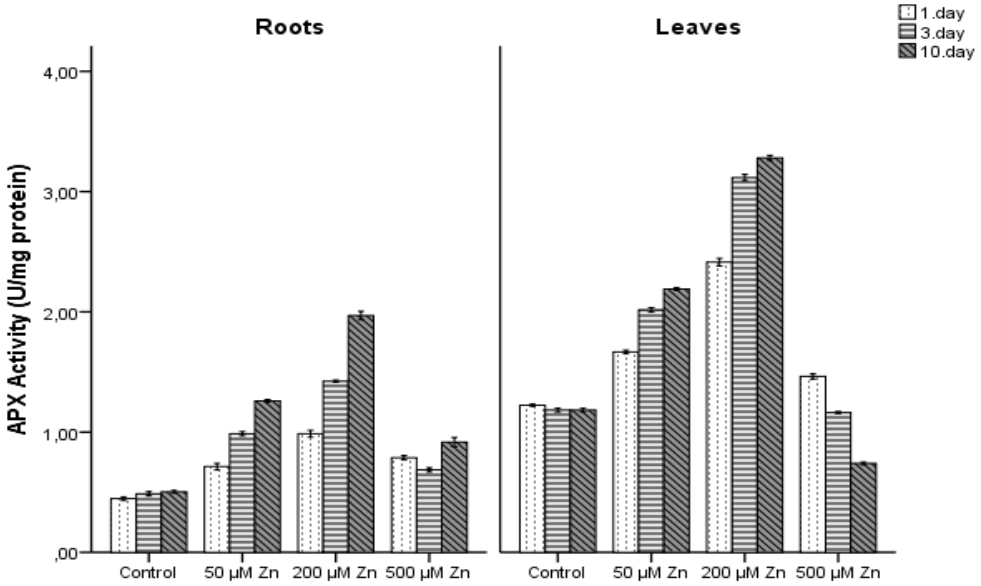

Figure 5. APX activity in the roots and leaves of *V. olympicum* seedlings exposed to different Zn concentrations for ten days. The values are the mean of five replicates ± SD. All the values are significant at *p<0*.05 compared to the control. Root APX Activity, *F*Concentration*(*3,48*)* = 683.104, *p* = .000, *F*Duration*(*2,48*)* = 251.097, *p* = .000, *F*Concentration x Duration*(*6,48*)* = 68.275, *p* = .000. Leaf APX Activity, *F*Concentration*(*3,48*)* = 2359.03, *p* = .000, *F*Duration*(*2,48*)* = 48.98, *p* = .000, *F*Concentration x Duration*(*6,48*)* = 134.80, *p* = .000.