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Antioxidative defense mechanism of the ruderal *Verbascum olympicum* Boiss. against copper (Cu)-induced stress

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Abstract: The endemic *Verbascum olympicum* has characteristics that allows it to live in degraded areas of Uludağ Mountain, Turkey and therefore known as a ruderal species. In this study, *V. olympicum* seeds collected from Uludağ Mountain were grown in the Hoagland nutrient solution, under hydroponic conditions. The activity of antioxidative enzymes (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX) were examined to demonstrate the role of antioxidative mechanism in the seedlings exposed to different Cu concentrations (0, 50, 250, 500 μM) for seven days. Also, certain growth parameters (such as the water content, biomass production, soluble protein), the level of lipid peroxidation, cell membrane injury and permeability were investigated. As a result, some toxic effects are observed following the application of 500 μM Cu, after the seedlings growing in 50 and 250 mM Cu concentrations showed high resistance and survived in hydroponic conditions. Our findings provide information about the resistance of *V. olympicum* seedlings to oxidative stress caused by excessive Cu concentrations.

Keywords: Copper, Superoxide dismutase, Ascorbate peroxidase, Catalase, *V. olympicum*

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1 Introduction

Destroyed areas such as the roadsides, developed building areas, rubbish tips, mining areas are increasing because of rapid industrialization and urbanization. It has become an increasingly serious environmental problem [1], as these disturbances give rise to heavy metal contamination of ecosystems.

Copper is the third most used metal in the world [2]. The upper limit of Cu content in unpolluted soil is 15 mg/kg [3]. When it is above this limit, it is a source of stress for many plant species, mostly ending with the death of the plant species [4]. Disruption of membrane permeability leading to ion leakage, inhibition of photosynthesis and respiratory processes, inhibition in enzyme activity and increased levels of oxidative stress are negative effects of high Cu concentrations in plants [5-7]. Also, excess Cu concentrations induce overproduction of reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) [8]. Antioxidative defence systems, including enzymes such as superoxide dismutase (SOD); catalase (CAT) and ascorbate peroxidase (APX), play an important role in preventing damage created by ROS. These protective capacity of the antioxidative defence systems change according to species, Cu concentrations and exposure durations [9].

Ruderal species typically dominant in degraded areas and have a relatively great potential to use limited resources in degraded areas. These plant species were endemic and have adapted to contaminated soils by developing tolerance mechanisms to metal-induced stress. *Verbascum olympicum* has become a dominant ruderal plant in the local flora of destroyed areas in Uludağ Mountain (Bursa-Turkey) [10]. At the same time, *V. olympicum* has some functional properties like high nitrate assimilation capacity [11] and high organic matter production [12]. In addition, heavy metal accumulation in different plant parts of *V. olympicum* have been determined by Güleryüz et al. [13,14].

In this study, we aimed to determine whether variations in antioxidant defence system play a role in the Cu tolerance of *V. olympicum* and to investigate the levels of oxidative damage caused by excess Cu concentrations as the Cu content in the destroyed areas of Uludağ Mountain is about twice the upper limit of unpolluted soil [15]. Thus, we investigated the effects of excess copper concentrations (50, 250 and 500 µM) in the *V. olympicum* seedlings growing under hydroponic conditions and identified both physiological and biochemical changes induced by culture in the presence of copper. We analyzed a number of plant growth parameters (such as the water content, biomass production and soluble protein), the cell membrane integrity and permeability, the level of lipid peroxidation and the activity of antioxidative enzymes (SOD, CAT and APX) in both leaves and roots of the seedlings.

2 Materials and Methods

2.1 Plant material and culture conditions

Seeds were collected from 1850-1900 m elevation of Uludağ Mountain, Turkey and were stored in paper bags in room conditions. To avoid variations between individual plants due to genetic characteristics, seeds from only one individual plant were used in the experiments. The seeds were sterilised with 5% sodium hypochlorite for 5 min and rinsed with double distilled water. Sterilised seeds were planted in Petri dishes with the help of water-moistened filter paper (Figure 1). Petri dishes were enclosed with aluminium foil for germination in dark conditions and incubated at 20°C for 48 h. For the development of cotyledons in germinating seeds, Petri dishes were wrapped with stretch film and placed in a growth chamber (Heraeus Vötsch HPS500) at 15°C/25°C day/night temperature with a 16 h photoperiod. On the 10th day after sowing, seedlings with two cotyledons were transferred to polyvinyl chloride plates floating on 10% Hoagland's nutrient solution [16], (Figure 2). This solution was renewed every other day and concentration of the solution was increased by 10% every week. The seedlings were grown in these conditions for eight weeks (Figure 3). Then uniform plants with 8-leaves were selected from among seedlings growing in the presence of Cu, which was added in the form of $CuSO_4$ (E. Merck, Darmstadt, Germany, 1027901000). Here, either 0 (control), 50, 250 or 500 µM Cu was added to 80% Hoagland nutrient solution. Five plants per treatment were used and three replications

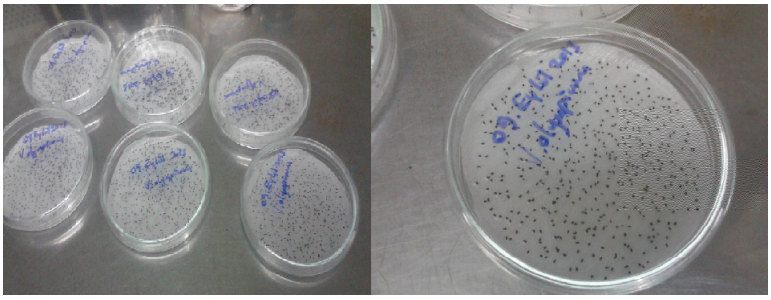

Fig. 1: The sowed seeds of *V. olympicum* in sterilised petri dishes.

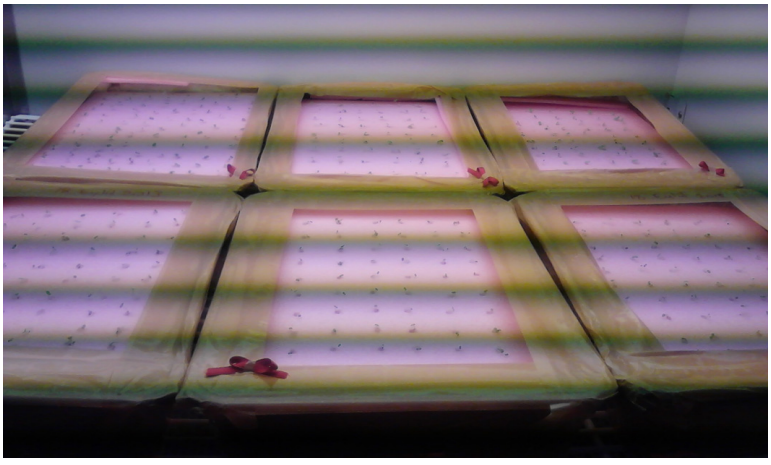

Fig. 2: The seedlings with two cotiledone in 10 % Hoagland solution.

Fig. 3: Eight week old seedlings, before Cu application.

per plant were performed. Plants were harvested on the 1st, 3rd and 7th days of treatment. Plant samples were washed thoroughly with de-ionised water and separated into root and leaf portions. Fresh plant materials were used for the determination of growth parameters such as the water content, biomass production and cell membrane injury and permeability; the rest were stored at -70°C after freezing in liquid nitrogen.

2.2 Plant biomass and water content

Plant biomass was measured on dry weight basis (mg dry weight / plant). Fresh weight of plant samples was measured. Then, they were dried at 80°C until the weight became constant and their dry weight was measured. Water content (%) and the biomass of roots and leaves were determined by assessing differences between fresh and dry weights.

2.3 Cell membrane injury and permeability

For an estimation of the cell membrane permeability, electrolyte leakage was determined according to the method described by Masood et al. [17]. Fresh leaves (0.5 g) and root samples (0.25 g) were cut into pieces and immersed in 20 ml distilled de-ionised water. They were incubated in a water bath at 32°C for 2 h and the initial electrical conductivity of the medium (EC_1) was measured. Then, the samples were autoclaved at 121°C for 20 min to release all the electrolytes. After cooling to 25°C, 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).

The cell membrane permeability (%): $(EC_1/EC_2)*100$

Also, cell membrane injury was expressed according to the formula above expressed as a percentage, [18]:

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 Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by thiobarbituric acid (TBA) reaction and was expressed as nmol/g fresh weight. MDA content was determined described by Heath & Packer [19] with some modifications. Leaf samples (0.1 g fresh weight) were homogenised 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 added into new tubes. After incubation at 95°C for 30 min, the tubes were cooled with ice and centrifuged (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 the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.5 Total soluble protein (TSP) content

TSP content was determined according to the method described by Bradford [20] and expressed as mg g^{-1} fresh weight. To prepare the stock dye solution, 100 mg

Coomassie Brillant Blue G250 was dissolved in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added. When the dye had completely dissolved, it was diluted to 1 litre and filtered through Whatman #1 paper. The dye solution was stored in a dark bottle at 4°C. BSA (bovine serum albumin) was used as a standard.

2.6 Extraction of plant material for antioxidative enzyme assay

1 g fresh mass was homogenised with 3 ml of buffer solution containing 50 mM Na-phosphate buffer (pH 7.8), 1 mM EDTA and 2% (w/v) polyvinylpolypyrrolidone (PVP) in an ice bath. Homogenised materials were centrifuged at 14,000 g for 40 min at 4°C. The supernatants were transferred into Eppendorf tubes for the determination of enzymatic activities. For the APX activity assay, 2 mM ascorbate was added to the extraction buffer solution described above.

2.7 Superoxide dismutase (SOD) assay

The SOD (EC 1.15.1.1) activity was determined by the method of Beauchamp & Fridovich [21]. This method is based on the inhibition of the nitroblue-tetrazolium at 560 nm. The total mixture (3 ml) in the SOD activity assay contained 0.5 ml of the enzyme extract, 2.5 ml of an ice-cold buffer containing 20 mM sodium phosphate buffer (pH 7.5), 0.1 mM EDTA, 10 mM methionine, 0.1 mM *p*-Nitro Blue Tetrazolium (NBT) and 5 µM riboflavin. Control test tubes (without samples) and sample test tubes were placed under 300 µmol m^{-2} s^{-1} fluorescent light for 15 minutes. Then, the reaction was stopped by switching off the light. The absorbance of the solution was measured at 560 nm (Novaspec II LKB Biochrom). The SOD assay kit (SOD S7446, Sigma-Aldrich, USA) was used in the preparation of standards. After the calculation of % inhibition, the enzyme activity was determined according to the linear equation which is obtained from the curve and expressed as units per mg protein.

2.8 Catalase (CAT) assay

The CAT (EC 1.11.1.6) activity was assayed as described by Lester *et al.* [22] with some modifications. The reaction was initiated by adding 0.1 ml of the enzyme extract to the assay mixture containing 20 mM sodium phosphate buffer (pH 6.8) and 15 mM H_2O_2. The change in absorbance was measured at 240 nm (extinction coefficient 40 $mM^{-1}cm^{-1}$) for 3 minutes (Shimadzu UV-2100). The activity of enzyme was expressed as units per mg protein.

2.9 Ascorbate peroxidase (APX) assay

The APX (EC 1.11.1.11) activity was determined according to the method described by Lester *et al.* [22]. This enzyme activity was stated from the decrease in absorbance at 290 nm as ascorbate was oxidised. The reaction mixture was comprised of 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 (extinction coefficient of 2.8 $mM^{-1}cm^{-1}$) for 3 minutes (Shimadzu UV-2100). The activity of enzyme was expressed as units per mg protein.

2.10 Statistical analysis

Results were expressed in figures and tables as mean values ± SD. The experiments were set up in a completely randomised design. According to different Cu concentrations and exposure periods, data were subjected to two-way ANOVA using SPSS 16.0 for Windows (SPSS Inc. 2007). For statistical analyses significance of the results was noted at $P < 0.05$ levels.

3 Results

3.1 Effects of Cu on plant growth and morphology

The growth of *V. olympicum* seedlings was decreased depending on the time of exposure to Cu and the concentration of Cu. The root and leaf biomass values began to decline on the 3rd day and reached a minimum value on the 7th day ($P < 0.05$). The lowest biomass values were measured in the seedlings exposed to 500 µM Cu on the 7th day for both the leaves (366.6 ± 24.5 mg dry weight / plant) and roots (158.8 ± 9.1 mg dry weight / plant) (Table 1). The water content (%) in the leaves and roots decreased with as growth conditions induced increasing Cu concentrations on all the sampling days ($P < 0.05$). Whereas the highest root and leaf water content was observed in control seedlings on the 7th day, the lowest root and leaf water content values were obtained in the seedlings exposed to 500 µM Cu on the 7th day (Table 1).

Table 1: Biomass (mg dry weight / plant) and water contents (%) in plant parts of *V. olympicum* seedlings exposed to different Cu concentrations for seven days (Mean ± Standard Deviation; n = 5, α = 0.05).

Biomass (mg dry weight/plant)					Water Contents (%)		
	Concentrations	**1st day**	**3rd day**	**7th day**	**1st day**	**3rd day**	**7th day**
Leaves	**Control**	696.0 ± 41.3	721.6 ± 30.1	764.5 ± 29.3	88.6 ± 7.3	87.2 ± 5.0	90.6 ± 5.4
	50 µM Cu	689.9 ± 35.5	663.3 ± 33.0	641.9 ± 37.9	88.0 ± 4.8	71.8 ± 3.3	64.6 ± 3.6
	250 µM Cu	672.8 ± 24.5	548.6 ± 29.5	513.6 ± 24.5	78.2 ± 3.0	57.6 ± 3.0	48.8 ± 2.3
	500 µM Cu	679.8 ± 25.7	463.6 ± 30.7	366.6 ± 24.5	69.2 ± 3.3	39.4 ± 2.4	23.8 ± 1.8
Roots	**Control**	364.9 ± 50.5	440.8 ± 24.7	449.8 ± 26.7	79.6 ± 3.8	79.6 ± 3.6	81.6 ± 2.6
	50 µM Cu	382.6 ± 25.3	347.7 ± 32.9	294.7 ± 28.1	79.6 ± 6.0	73.0 ± 2.0	70.2 ± 1.8
	250 µM Cu	357.2 ± 26.6	280.6 ± 15.1	191.2 ± 28.9	78.8 ± 3.6	64.4 ± 3.4	47.8 ± 3.2
	500 µM Cu	360.7 ± 38.2	226.9 ± 22.3	158.8 ± 9.1	77.6 ± 3.8	50.4 ± 2.6	28.4 ± 2.7

[All parameters were analysed for concentration (Cu) × duration at α; 0.05 significance level according to Two-way ANOVA. **Root Biomass**, $F_{Concentration\ (3,48)} = 100.79$, $p = 0.000$, $F_{Duration\ (2,48)} = 50.69$, $p = 0.000$, $F_{Concentration \times Duration\ (6,48)} = 25.39$, $p = 0.000$. **Leaves Biomass**, $F_{Concentration\ (3,48)} = 150.15$, $p = 0.000$, $F_{Duration\ (2,48)} = 72.09$, $p = 0.000$, $F_{Concentration \times Duration\ (6,48)} = 35.70$, $p = 0.000$. **Root Water content**, $F_{Concentration\ (3,48)} = 194.19$, $p = 0.000$, $F_{Duration\ (2,48)} = 203.33$, $p = 0.000$, $F_{Concentration \times Duration\ (6,48)} = 55.14$, $p = 0.000$. **Leaves Water** content, $F_{Concentration\ (3,48)} = 327.02$, $p = 0.000$, $F_{Duration\ (2,48)} = 183.69$, $p = 0.000$, $F_{Concentration \times Duration\ (6,48)} = 29.54$, $p = 0.000$].

In addition to growth restriction, chlorosis occurred in the margins of the leaves of the seedlings and a reduction in the expansion of leaves was observed at 500 mM Cu treatment (Figure 4). Also, the formation of roots in the seedlings exposed to 500 mM Cu treatment on the 7th days was poor, resulting in decreased root length and root volume. In the plants treated with 50 mM Cu only root length reduced (Figure 5).

The total soluble protein content was significantly decreased in the roots and leaves of seedlings treated with 250 µM and 500 µM Cu (Figure 6; $P<0.05$). The lowest TSP content was found in the 500 µM Cu-treated seedlings at 7 days for both the leaves (1.30 ± 0.03 mg/g YA) and roots (1.01 ± 0.04 mg/g YA). However, the TSP content was increased in the roots and leaves of seedlings with 50 µM Cu treatment (Figure 6). The highest TSP content in the seedlings treated with 50 µM Cu was found on the 7th day for the roots and on the 3rd day for the leaves.

3.2 Changes in MDA (lipid peroxidation) content and cell membrane injury and permeability

The MDA contents in the leaves of seedlings increased markedly when growth conditions included Cu concentrations at 250 µM and 500 µM (Figure 7; $P < 0.05$). The highest MDA content was determined in the leaves of

Fig. 4: The 500 µM Cu-treated seedlings on the 7th day.

Fig. 5: The 50 µM Cu-treated seedlings on the 7th day.

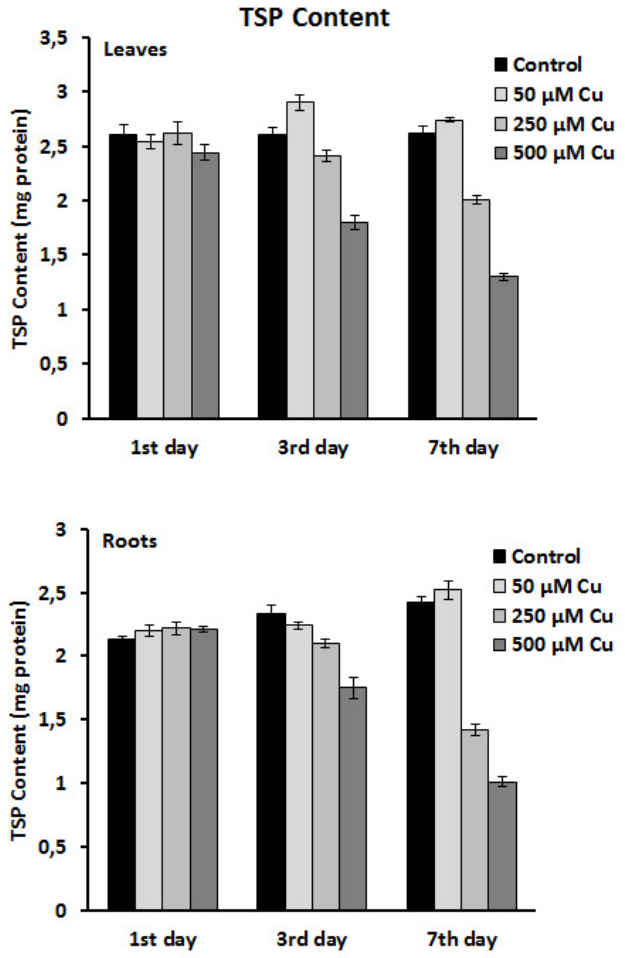

Fig. 6: Total Soluble Protein (TSP) contents in leaves and roots of *Verbascum olympicum* seedlings exposed to different Cu concentrations for seven days (mg protein, n = 5). Values are mean of five replicates ±SD and bars indicate standart error (SE). [All parameters were analysed for concentration (Cu) × duration at α; 0.05 significance level according to Two-way ANOVA. **Root TSP Content**, $F_{Concentration\ (3,48)} = 640.62$, p = 0.000, $F_{Duration\ (2,48)} = 269.91$, p = 0.000, $F_{Concentration \times Duration\ (6,48)} = 328.31$, p = 0.000. **Leaves TSP Content**, $F_{Concentration\ (3,48)} = 499.92$, p = 0.000, $F_{Duration\ (2,48)} = 166.58$, p = 0.000, $F_{Concentration \times Duration\ (6,48)} = 111.09$, p = 0.000].

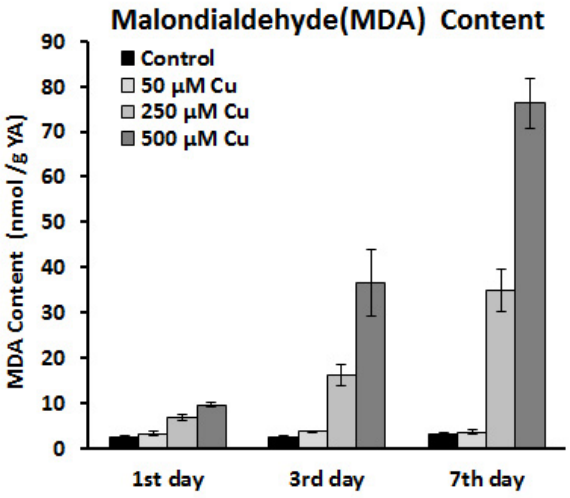

Fig. 7. Malondialdehyde (MDA) contents in leaves of *V. olympicum* seedlings exposed to different Cu concentrations for seven days (nmol/gYA, n = 5). Values are mean of five replicates ±SD and bars indicate standart error (SE). [All parameters were analysed for concentration (Cu) × duration at α; 0.05 significance level according to Two-way ANOVA. **Leaves MDA Content**, $F_{Concentration\ (3,48)} = 512.33$, p = 0.000, $F_{Duration\ (2,48)} = 312.38$, p = 0.000, $F_{Concentration \times Duration\ (6,48)} = 132.8$, p = 0.000].

the seedlings exposed to 500 µM Cu for 7 days. However, in the seedlings treated with 50 µM Cu, no changes relating to lipid peroxidation were observed.

Electrolyte leakage, which was representative of the cell membrane permeability, tended to increase significantly in both the leaves and roots with increasing Cu concentrations and increasing durations of exposure (Table 2; P < 0.05). The percentage of electrolyte leakage at 500 µM Cu concentration on the 7th day exhibited the highest value for both the leaves and roots. The highest electrolyte leakage value in the roots was approximately 30% higher than the value of the control seedlings on the 7th day, whereas electrolyte leakage approximately doubled in the leaves compared to the control seedlings after 7 days of treatment.

A similar trend was also observed for the cell membrane injury and this level of injury increased in both the leaves and roots with exposure to increasing levels of Cu on all sampling days (Table 2; P < 0.05). The highest value was found in the seedlings exposed to 500 µM Cu treatment for 7 days. This value was 75% for the leaves, 65% for the roots.

3.3 Alteration in the antioxidant enzymes activities

The SOD enzyme activity in both the roots and leaves was elevated by growth conditions with added Cu; this elevation of SOD enzyme activity was proportional to the increasing Cu concentrations and durations of exposure (Figure 8; P < 0.05). The highest SOD activity was determined in the leaves and roots of seedlings exposed to 500 µM Cu for 7 days. Whereas this value for the roots was approximately 6 times higher than the control seedlings; for the leaves, it was approximately 3 times higher than the control seedlings.

The Cu treatments led to remarkable elevation of the APX activity in both the leaves and roots (Figure 8). The APX activity was significantly increased in the

Table 2: The electrolyte leakage (%) and cell membrane injury (%) in plant parts of *V. olympicum* seedlings exposed to different Cu concentrations for seven days (Mean ± Standard Deviation; n = 5, α = 0.05).

		Electrolyte leakage (%)			Cell Membrane Injury (%)		
	Concentrations	**1st day**	**3rd day**	**7th day**	**1st day**	**3rd day**	**7th day**
Leaves	**Control**	44.2 ± 3.0	46.7 ± 3.1	43.6 ± 2.4	0	0	0
	50 µM Cu	66.8 ± 1.9	69.4 ± 1.1	69.2 ± 2.1	37.8 ± 3.4	45.8 ± 2.0	45.5 ± 3.7
	250 µM Cu	69.6 ± 1.4	74.6 ± 1.8	81.5 ± 1.9	46.3 ± 2.4	55.0 ± 3.1	67.3 ± 3.3
	500 µM Cu	74.3 ± 1.4	79.9 ± 1.6	86.0 ± 2.1	54.6 ± 2.6	64.5 ± 2.9	75.2 ± 3.8
Roots	**Control**	54.5 ± 3.2	54.4 ± 1.6	53.7 ± 2.9	0	0	0
	50 µM Cu	63.2 ± 0.5	64.7 ± 0.9	67.0 ± 0.3	19.2 ± 1.0	22.5 ± 1.9	27.6 ± 0.8
	250 µM Cu	66.8 ± 0.5	70.9 ± 1.1	74.6 ± 0.8	27.3 ± 1.0	36.2 ± 2.4	44.3 ± 1.8
	500 µM Cu	69.5 ± 0.8	76.6 ± 1.1	84.1 ± 1.1	33.2 ± 1.9	48.7 ± 2.3	65.2 ± 2.4

[All parameters were analysed for concentration (Cu) × duration at α; 0.05 significance level according to Two-way ANOVA. **Root Electrolyte leakage percentage**, $F_{Concentration\ (3,48)} = 606.89$, p = 0.000, $F_{Duration\ (2,48)} = 87.46$, p = 0.000, $F_{Concentration\ x\ Duration\ (6,48)} = 23.42$, p = 0.000. **Leaves Electrolyte leakage percentage**, $F_{Concentration\ (3,48)} = 851.31$, p = 0.000, $F_{Duration\ (2,48)} = 55.70$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 12.06$, p = 0.000. **Root Cell Membrane Injury percentage**, $F_{Concentration\ (3,48)} = 2592.07$, p = 0.000, $F_{Duration\ (2,48)} = 406.28$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 92.7$, p = 0.000. **Leaves Cell Membrane Injury percentage**, $F_{Concentration\ (3,48)} = 1735.23$, p = 0.000, $F_{Duration\ (2,48)} = 106.82$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 20.08$, p = 0.000].

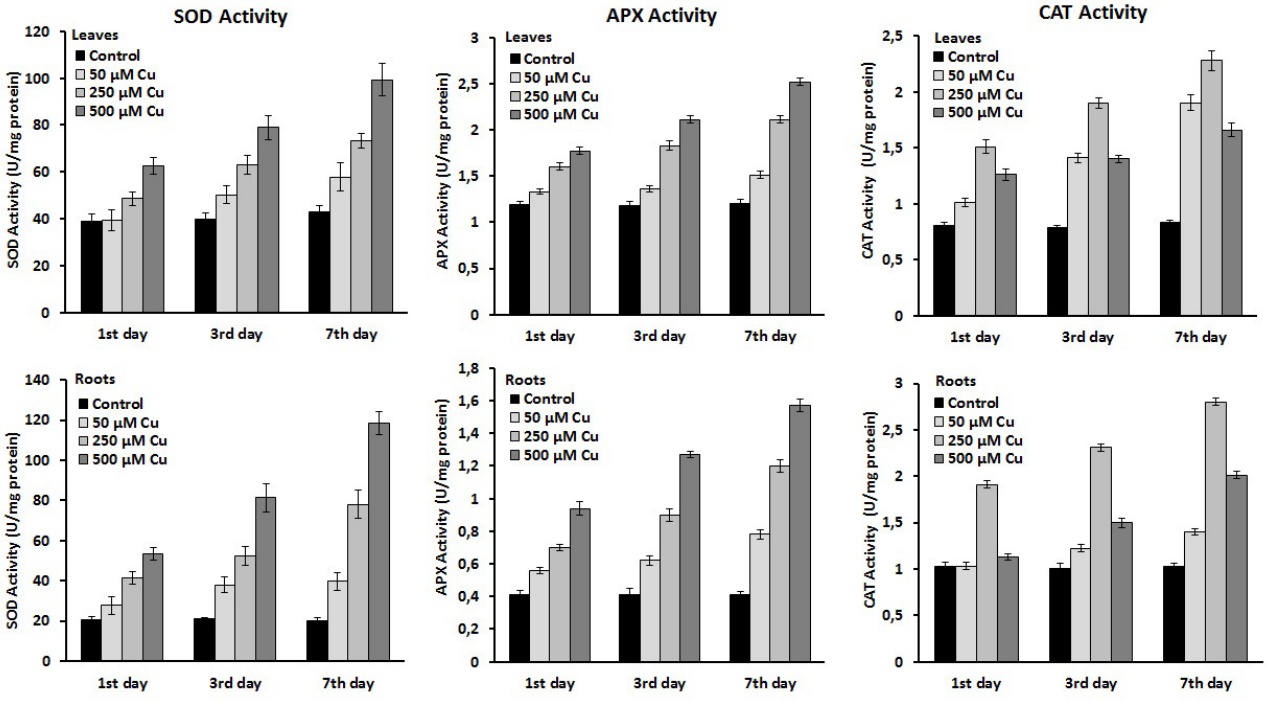

Fig. 8: Enzyme activities of Superoxide dismutase (SOD), Askorbate peroxidase (APX) and Catalase (CAT) in leaves and roots of *V. olympicum seedlings* exposed to different Cu concentrations for seven days (U/mg protein, n = 5). Values are mean of five replicates ±SD and bars indicate standart error (SE). [All parameters were analysed for concentration (Cu) × duration at α; 0.05 significance level according to Two-way ANOVA. **Root SOD Activity**, $F_{Concentration\ (3,48)} = 594.48$, p = 0.000, $F_{Duration\ (2,48)} = 204.57$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 56.52$, p = 0.000. **Leaves SOD Activity**, $F_{Concentration\ (3,48)} = 246.71$, p = 0.000, $F_{Duration\ (2,48)} = 121.93$, p = 0.000, $F_{Concentration\ x\ Duration\ (6,48)} = 13.13$, p = 0.000. **Root APX Activity**, $F_{Concentration\ (3,48)} = 1767.20$, p = 0.000, $F_{Duration\ (2,48)} = 516.54$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 89.95$, p = 0.000. **Leaves APX Activity**, $F_{Concentration\ (3,48)} = 1707.99$, p = 0.000, $F_{Duration\ (2,48)} = 409.64$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 91.73$, p = 0.000. **Root CAT Activity**, $F_{Concentration\ (3,48)} = 2943.23$, p = 0.000, $F_{Duration\ (2,48)} = 829.50$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 137.60$, p = 0.000. **Leaves CAT Activity**, $F_{Concentration\ (3,48)} = 1208.40$, p = 0.000, $F_{Duration\ (2,48)} = 544.20$, p = 0.000, $F_{Concentration\ \times\ Duration\ (6,48)} = 76.13$, p = 0.000].

roots and leaves of seedlings depending on both the Cu concentration and duration of exposure ($P < 0.05$). The mean APX activity reached 2.52 ± 0.04 units /mg protein in the leaves of seedlings treated with the highest Cu concentration on the 7th day, whereas it was 1.20 ± 0.05 units /mg protein in control seedlings on the first day. A similar trend was also observed for the APX activity for the roots samples. The highest APX activity was observed in the roots of seedlings exposed to 500 µM Cu for 7 days.

The leaf and root CAT activity of seedlings varied significantly with treatments of increasing Cu concentrations and increasing durations of exposure (Figure 8; $P < 0.05$). The highest values for CAT activity were observed on the 7th day with growth conditions of 250 µM Cu for both leaves and roots. This value reached 2.80 ± 0.04 units /mg protein for the roots of seedlings and 2.28 ± 0.09 units /mg protein for the leaves. The CAT activity in both the roots and leaves of seedlings decreased when growth conditions included 500 µM Cu.

4 Discussion

These findings indicate that the water content (%) and biomass value (mg dry weight / plant) in the leaves and roots of *V. olympicum* were significantly decreased by addition of increasing levels of Cu in the nutrient medium (Table 1). It is seen that the growth of *V. olympicum* seedlings slowed due to increasing Cu concentrations and duration of exposure to elevated Cu concentrations. Plant growth and development are negatively affected by oxidative stress, which presumably occurred due to the generation of large amounts of ROS induced by the treatments with elevated Cu concentrations. Moreover, it is known that oxidative stress damages a range of specific plant hormones that play important roles in the growth and development [23].

In addition to growth restriction, chlorosis and poor root formation occurred in the margins of leaves of seedlings exposed to 500 mM Cu treatment indicating that the seedlings of *V. olympicum* are negatively affected by elevated Cu concentrations (Figure 4). It is possible that these observed changes in the seedlings may be due to competition between elevated Cu ions and other metal ions present in the nutrient solution, such as Mn, Zn, and Fe, which serve as micronutrients [24]. Indeed, it has been reported that similar growth related changes in the plant morphology occur due to Fe deficiency [25]. These effects resulted in a decrease of the water content of plants due to a reduction in the absorption surface and similar observations have been reported by studies using radishes [25] and sunflowers [26].

Growth of plants in the presence of elevated levels of Cu is able to directly generate ROS through the Haber-Weiss reaction and the increased levels of ROS initiate oxidative stress-related effects [27, 28]. Lipids and proteins are the main target of ROS, such as H_2O_2, $OH^{\bullet}$ and $O_2^{-\bullet}$ and lipid peroxidation is the most obvious indicator of oxidative stress. For example, when the seedlings of *V. olympicum* were treated with 250 µM and 500 µM Cu in the nutrient medium marked increase in malondialdehyde (MDA) content were observed; these effects were also dependent on treatment duration (Figure 7). However, in the 50 µM Cu treatments, no such changes were observed. The increased capacity of lipid peroxidation observed in treatments with ≥ 250 µM Cu is probably due to the oxidizing properties of Cu^{+2}, which promotes the catalytic formation of extremely reactive hydroxyl radicals [29]. Similar results have also been reported in leaves of *Peganum harmala* [30], *Withania somnifera* [31] and *Astragalus neo-mobayenii* [32]. Furthermore, Olga et al. [33] reported that elevated levels of copper stimulated lipid peroxidation and enhanced membrane permeability in *Hydrilla verticillata*.

Electrolyte leakage which is representative of cell membrane permeability is in accordance with the findings in MDA content (Table 2). Electrolyte leakage was markedly increased as a function of Cu treatment in our study. The obtained results in cell membrane injury were similar to increase in the level of electrolyte leakage. The highest value in the cell membrane injury was found in the leaves and roots of seedlings exposed to 500 µM Cu for 7 days. In support of these current findings it has been found that electrolyte leakage was increased by the generation of ROS due to decreasing the membrane stability in *Avicennia germinans* and *W.somnifera* [31, 34].

The TSP content is another important indicator in understanding the responses to metal induced-stress responses [35] and these effects occur because ROS generation induced by heavy metals causes disturbances in protein synthesis pathways. In the current study, treatment in the presence of 50 µM Cu caused an increase in the TSP content. These results suggest that these growth conditions in the presence of 50 µM Cu was not the cause of structural damage in *V. olympicum* seedlings in the short-term. However, TSP content in all plant parts (leaves and roots) remarkably decreased in treatments with 250 µM Cu (Figure 6). These alterations are related to nitrogen assimilation and also to the inhibition of enzymes involved in protein synthesis in *Verbascum olympicum* [14]. Similar results have been observed in Cu-tolerant *E. haichowensis* [36] and also in both tolerant and sensitive species of *Silene vulgaris* [37].

Boojar and Goodarzi [27] reported that increases in antioxidative enzyme activities in plants is related to the level of tolerance against Cu toxicity. In this current study, growth condition including elevated Cu concentrations increase the antioxidative enzyme activity. The levels of SOD activity in both the roots and leaves are directly related to both the increasing Cu concentrations and durations of exposure (Figure 8). The highest SOD activity in all plant parts was found in the seedlings exposed to 500 μM Cu for seven days. Since SOD acts as a primary agent in defence against oxidative stress, the changes observed herein emphasise that exposure to Cu induces stress responses that generate superoxide radicals in *V. olympicum* seedlings. Furthermore, our observations are consistent with those advanced by Thounaojam *et al.* [38] and Devi & Prasad [39]. In this current study, the levels of SOD activity in the roots was greater than that in the leaves of *V. olympicum* seedlings suggesting that there is a higher level of oxidative stress in roots.

The Cu treatments led to a remarkable elevation in APX activity in both the leaves and roots (Figure 8). The increased levels of APX activity in *V. olympicum* seedlings indicates the importance of the ascorbate-glutathione (ASH-GSH) cycle in reducing the toxic effect of elevated Cu concentrations [40]. Thounaojam *et al.* [38] reported that elevated APX activity acts to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organelles and enhancement of APX activity has been observed in various plants such as rice, tea and mulberry under different treatment conditions with elevated Cu concentrations [38, 41, 42]. In addition to these changes in APX activity, the CAT activity in both the roots and leaves of *V. olympicum* seedlings decreased in growth condition with 500 μM Cu concentration (Figure 8). The present study showed that CAT enzyme activity in *V. olympicum* seedlings is sensitive to high Cu concentrations and its activity is inhibited in treatments with such high Cu concentrations. In fact, it has been reported that elevated Cu levels have an inhibitory effect on CAT activity [43]. Similar responses in CAT activity have also been described in several studies [44-46].

5 Conclusion

Responses of *V. olympicum* to Cu-induced stress are important indicators for reversible and irreversible changes in metabolic homeostasis. In this study, the effect of elevated Cu concentrations was investigated due to the fact that *V. olympicum* is a species that can live in degraded areas. The *V. olympicum* seedlings growing in the presence of 50 and 250 mM Cu concentrations were largely resistant to the potential adverse toxic effects of Cu and survived under these hydroponic growth conditions. It is important to note that at Cu concentrations of 50 mM no negative effects on the growth parameters and cell membrane integrity were observed. The long-term effects of the growth conditions including 250 μM Cu is a research topic for future studies. Furthermore, treatments with 500 mM Cu concentrations for seven days appeared to be the most toxic conditions for *V. olympicum* seedlings and all measured parameters reached maximal levels. Our current findings suggest that *V. olympicum* seedlings exhibit resistance to oxidative stress promoted by elevated Cu concentrations. It is proposed that the resistance to Cu-induced stress demonstrated by *V. olympicum* seedlings is related to the plants ability to maintain a balance between the generation of ROS and their detoxification by SOD, CAT and APX.

Previous studies investigating the physiological and antioxidative responses to heavy metal toxicity have generally been on cultivated plants [1,5,39,47]. There are very few studies that investigate Cu-induced stress responses in wild plants. However, from an ecological perspective it is increasingly important to understand the mechanisms that maintain the life of wild plants due to the increasing problems associated with metal pollution worldwide.

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