Antioxidant Responses of *Helianthus annuus* L. under Vanadium Stress

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Abstract

Vanadium (V) is a transition metal consistently distributed in the Earth's crust. At trace concentrations, it has been described as a beneficial element for some organisms; however, it is toxic at higher concentrations. This study was conducted hydroponically in a complete randomized design to investigate effects of V (0, 3.25, 7.5, and 15 mg L^{-1}) on antioxidant system of sunflower. Results showed that all applied concentrations of V significantly decreased the plant growth, but increased the free amino acids, proline, malondialdehyde and H_2O_2 contents of plants. The antioxidant enzymes activities increased in response to the increase in V concentration. The activity of (Guaiacol) peroxidase showed a significant increase at 15 mg L^{-1} V while the activities of catalase, glutathione reductase, and ascorbate peroxidase decreased significantly by application of V. There was a slight increase in the superoxide dismutase activity at different concentrations of V. The study concludes that the applied concentrations of V induced oxidative stress in sunflower and (Guaiacol) peroxidase was a more effective antioxidant enzyme in scavenging the free radicals in this plant.

Keywords: *Helianthus annuus*, antioxidant enzymes, free amino acids, H_2O_2, MDA, proline

Abbreviations: ASX: ascorbate peroxidase; CAT: catalase; GR: glutathione reductase; MDA: malondialdehyde; POD: guaiacol peroxidase; PVP: polyvinylpyrrolidone; SOD: superoxide dismutase; V: vanadium

Abedini, M., F. Mohammadian, B. Daie-Hassani and **L. Zarandi-Miandoab.** 2017. 'Antioxidant Responses of *Helianthus annuus* L. Under Vanadium Stress '. *Iranian Journal of Plant Physiology* 8 (1): 2239-2245.

Introduction

Plants are the main source of food and energy for living organisms. The occurrence and concentration of different elements in the rooting zone of the soil affect the quality and quantity of plants' production. Vanadium (V) as an unstable element is increasingly released in the environment by the leaching of rocks, the combustion of coal or petroleum products, the pollution resulted from fertilizers, and residual slags from the steel manufacturing (Ringelb and and Heh, 2000). Vanadium is absorbed by plants as Vanadate (VO_3^-). The absorbed VO_3^- is partially reduced to VO_2^+ in roots or is chelated (Pilbeam, 2015). The concentration of vanadium in roots in various plant species is often more than its concentration in shoots. Vanadium at low

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Received: March, 2017
Accepted: September, 2017

concentrations is reported to stimulate growth of plants, enhancing chlorophyll synthesis, nitrogen fixation, and also utilization of potassium (Imtiaz et al., 2015). At higher concentrations, vanadium acts as a toxic element and by excessive reactive oxygen species (ROS) production induces oxidative stress in plants (Vachirapatama et al., 2011). When the subtle equilibrium between ROS production and removal, which is necessary for normal cellular homeostasis, is disturbed, the cellular damages are manifested in the form of biomolecules degradation. ROS detoxifying system is composed of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) as well as non-enzymatic antioxidants like ascorbate, reduced glutathione, carotenoids, alkaloids, α-tocopherols, proline, and phenolic compounds (flavonoids, tannins, and lignin) that act as the scavengers of free radicals (Nakabayashi et al., 2014).

Sunflower (*Helianthus annuus* L.) is an annual plant which is important for its oil worldwide. It is ranked as the 3rd important vegetable oil crop after soybean and rapeseed. The aim of this research was to study the effects of vanadium on some antioxidant enzymes and metabolites of sunflower plants.

Materials and Methods

Plant growth condition and analysis

The seeds of sunflower (*Helianthus annuus* cv. Dorsefid) were germinated in petri dishes under 15/10 °C temperature regime, and then transferred to 50% Hoagland solution for 7 days, followed by a full strength Hoagland solution. Vanadium (as VO_3NH_4; 0, 3.25, 7.5 and 15 mg L^{-1}) was applied 7 days after adapting to the full strength nutrition. During the seedling growth period, the nutrient solution was replaced every 6 days and the pH was set on 5.8 every two days. Photoperiod was 14 hours; light intensity was 195 µmol s^{-1} m^{-2} supplied by 10 fluorescent lamps and temperature regime was 25/18 °C. Plants were harvested 15 days after application of V. Three samples of each treatment were selected for dry weight assessment and the others were kept frozen in liquid nitrogen until analyses.

Antioxidant enzyme assays

To obtain the crude extract, 0.5 g of leaves were homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7) containing 0.2% PVP. The homogenate was centrifuged at 12000 g at 4 °C for 20 min. The supernatant was used to measure the activities of antioxidant enzymes and total protein content.

The activity of SOD was measured according to its capacity to inhibit photochemical reduction of nitrobluetetrazolium (NBT). The reaction mixture contained 2.65 mL of 67 mM potassium phosphate buffer (pH 7.8), 0.2 mL of 0.1 mM EDTA solution containing 0.3 mM sodium cyanide, 0.1 mL of 1.5 mM NBT, 50 mL of 0.12 mM riboflavin, and 0.5 µL of enzyme extract. The amount of enzyme that catalyzed 50% inhibition from photochemical reduction of NBT was defined as one unit of SOD (Winterbourn et al., 1976).

Guaiacol peroxidase (POD) was assayed following the method of Chance and Maehly (1955). The reaction mixture contained 1.5 mL of 100 mM citrate-phosphate- borate buffer solution (pH 7.5), 50 µL of 15 mM guaiacol, 25 µL enzyme extract and 50 µL of 3.3 mM H_2O_2. Polymerization of guaiacol was initiated by adding H_2O_2 and an increase in absorbance at 470 nm was recorded. POD activity was calculated using the extinction coefficient, 26.6 mM^{-1} cm^{-1}, for tetraguaiacol.

The activity of APX was measured according to Nakano and Asada (1987). The reaction mixture contained 25 µL of enzyme extract with 2.5 mL of phosphate buffer (pH 7) containing EDTA 0.1 mM, H_2O_2 1 mM, and ascorbic acid 0.25 mM. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of oxidized ascorbate was calculated using extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

The CAT activity was determined by monitoring the decomposition of H_2O_2 at 240 nm. The reaction mixture contained 1.5 mL of 100 mM citrate-phosphate-borate buffer solution (pH 7.5), 50 µL enzyme extract and 13 µL of 10 mM H_2O_2.

CAT activity was calculated using the extinction coefficient 39.4 mM^{-1} cm^{-1} (Obinger et al., 1997).

The activity GR was determined by measuring oxidation of NADPH at 340 nm using the extinction coefficient 220 M^{-1} cm^{-1} (Foyer and Halliwell, 1976). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH, and 50 µL enzyme extract.

Proline assays

Proline contents of samples were assayed using the method of Bates et al. (1973). The proline was extracted with 10 mL of 3% sulphosalycilic acid solution. 2 mL of the liquid was reacted with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid for 1 h in 100 °C and the reaction was terminated at ice bath. The reaction mixture was extracted by 4 mL toluene. The absorbance of chromophore containing toluene was read at 520 nm. Proline concentration of samples was determined from a standard curve.

Free amino acid assay

Free amino acids were extracted by 80% ethanol and centrifuged at 5000 g for 10 min. Samples were taken into test tubes and 1 mL of ninhydrin regeant and 0.2 mL of citrate buffer were added to them. The mixtures were incubated at 100 °C in a water bath for 10 min. The absorbance of samples was measured at 570 nm and free amino acids concentrations were calculated using a standard curve (Yemm and Cocking, 1955).

Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) assays

To obtain the crude extract, 0.1 g of leaves were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g at 4 °C for 15 min.

To assay the H_2O_2 content 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM phosphate buffer (pH 7) and 1 mL of 1 M potassium iodide. The mixture was incubated at 25 °C for 15 min. The absorbance was measured at 390 nm. The H_2O_2 content was calculated from a standard curve prepared in a similar way (Harinasut et al., 2003).

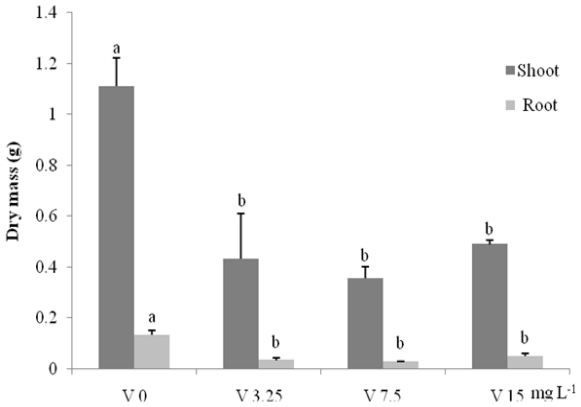

Figure I. Effects of vanadium on dry mass in sunflower

To assay MDA concentration, 1 mL of the supernatant was mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95 °C in a water bath for 30 min, and then quickly cooled on ice. The mixture was centrifuged at 10000 g for 15 min and the absorbance was measured at 532 nm. MDA levels were calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

All chemicals and reagents used in the experiment were purchased from Sigma Aldrich (Steinheim, Germany), Fluka (Steinheim, Germany) and Merck (Darmstadt, Germany).

Statistical Analysis

All assays were carried out in triplicate and the results were presented as mean values ± SD. Statistical analyses were performed using a one-way analysis of variance test and the significance of the difference between means was determined by Student's multiple range test. The InStat (3.0) software and Microsoft Excel package were used to perform statistical analyses.

Results

Growth Parameters

In sunflower plants, vanadium application at all concentrations significantly ($p<0.05$) decreased the dry masses of roots and shoots (Fig. I).

Table 1
Effects of V concentrations on some metabolites in leaves of sunflower

Treatment	H_2O_2 (μMg^{-1} FW^{-1})	MDA (nM g^{-1} FW^{-1})	Proline(mg g^{-1} FW^{-1})	Free amino acids (mg g^{-1} FW^{-1})
V 0 (control)	0.06±0.02c	0.017±0.0008b	0.16±0.002b	0.41 ± 0.04 c
V 3.25 mg L^{-1}	0.31±0.004a	0.019±0.0005b	0.23±0.001a	0.90 ± 0.03 a
V 7.5 mg L^{-1}	0.18±0.02b	0.025±0.001a	0.22±0.004a	0.61 ± 0.04 b
V 15 mg L^{-1}	0.15±0.01b	0.024±0.001a	0.22±0.001a	0.39 ± 0.01 c

Values within a column followed by different letters are significantly different ($p<0.05$, means ± SE).

Proline and free amino acids

Proline increased significantly in response to V application at all applied concentrations (Table 1). There was a significant increase in the free amino acid content of plants at concentrations of 3.25 and 7 mg L^{-1} of V, but application of 15 mg L^{-1} of V had no significant effect on this parameter (Table 1).

MDH and H_2O_2

According to the results obtained from this study, H_2O_2 and MDA contents of sunflower plants increased by V application. The induced increases in H_2O_2 content were significant at all applied concentrations of V. But, induced increases in MDA content were significant at 7.5 and 15 mg L^{-1} of V (Table 1).

Antioxidant enzyme activities

Vanadium application in sunflower caused a non-significant increase in the activity of SOD while it significantly decreased the activities of CAT and APX (Figs. II, III, IV). The activity of POD significantly increased at concentration of 15 mg L^{-1} of V while it was not affected at lower concentrations (Fig. V). The activity of GR was not affected significantly at concentrations of 3.25 mg L^{-1}of V, but it decreased significantly at the other concentrations of the study (Fig. VI).

Discussion

Vanadium application decreased the dry mass of sunflower plants in the study. The inhibition of plants' growth by V has been reported for some plant species (Vachirapatama et al., 2011). For example, this element inhibited the growth of soybean at concentration of 30 mg L^{-1} (Wang and Liu, 1999) and tomato at

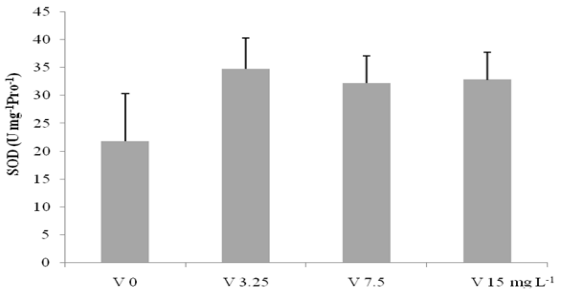

Figure II. Effects of vanadium on SOD activity in sunflower

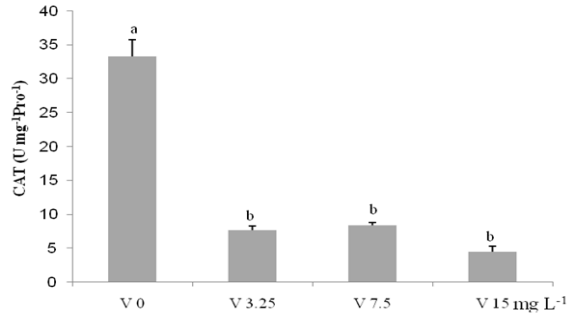

Figure III. Effects of vanadium on CAT activity in sunflower

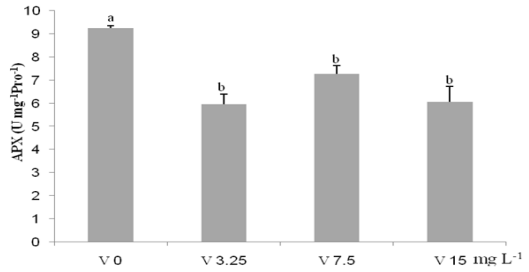

Figure IV. Effects of vanadium on APX activity in sunflower

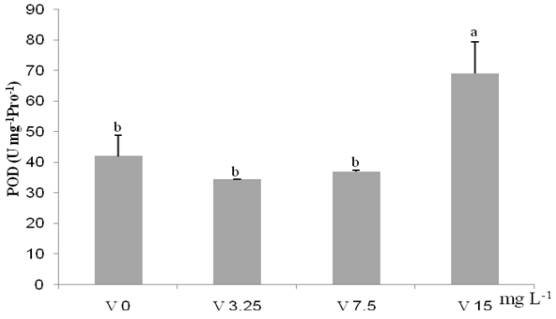

Figure V. Effects of vanadium on POD activity in sunflower

concentration of 40 mg L^{-1} (Vachirapatama et al., 2011). The induced obstruction of water and mineral absorption by V probably is responsible for the restricted growth of plants under V treatment (Vachirapatama et al. 2011). There are numerous indications concerning with antagonistic effects of V with essential nutrients such as Mg, Ca, and P (Olness et al., 2005). Vanadium interacts with these elements and inhibits the protein production, ATP synthesis, and enzymes activities (Imtiaz et al., 2015; Pilbeam, 2015). Moreover, V can be reduced easier than phosphorus (P) and can be converted into vanadyl form and vanadate and phosphate are chemical analogues. Therefore, this element potentially inhibits the enzyme reactions (Imtiaz et al., 2015). It has also been reported that V induces Fe deficiency in soybean plants (Nakanishi, 2001) and decreases the concentration of K in rice (Bucker et al., 2006) and Mo, Mn, Ni and Cu contents in *Sinapis alba* (Fargasova and Beinrohr, 2001). Similarly, the roles of vanadium were reported in induction of the stomata closure (Gepstein et al., 1982), ethylene production (Ching, 1996) and inhibition of the H^+-ATPase activity (Imtiaz et al., 2015).

Proline and free amino acids

The proline and free amino acid contents of sunflower increased in response to V application. Accumulation of low molecular mass compounds such as free amino acids ensures the osmotic adjustment of the plants (Good and Zaplachinski, 1994). It seems that the hydrolysis of soluble proteins to amino acids happened for this purpose. The role of proline accumulation as an adaptation mechanism to the wide range of biotic and abiotic stresses has been shown in different plant species. Moreover, large increase in the proline precursors such as glutamate (Glu) and arginine (Arg) has been reported during stresses (Good and Zaplachinski, 1994). In *Brassica napus*, drought induced a more than 5-fold increase in isoleucine, leucine, and aspartate contents (Good and Zaplachinski, 1994). Similarly, water deficit resulted in the accumulation of isoleucine, leucine, threonine, alanine, and valine in bean (Raggi, 1994).

MDH and H_2O_2

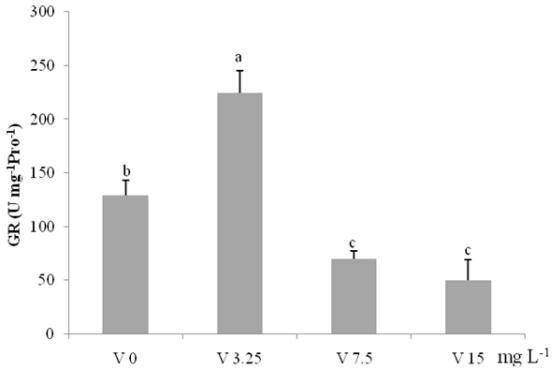

Figure VI. Effects of vanadium on GR activity in sunflower

Vanadium application increased the H_2O_2 and MDA contents of sunflower plants. Different biotic and abiotic stresses induce oxidative stress and formation of ROS (Kachout et al., 2009; Pandey et al., 2009). Vanadium compounds could generate reactive oxygen species as a result of Fenton's reaction or of the reaction with atmospheric oxygen, which induces lipid peroxidation (Lin et al., 2009). The increased ROS levels in plants can cause oxidative damage to biomolecules such as lipids thus increasing the MDA content as the decomposition product of polyunsaturated fatty acids of membranes (Heath and Packer, 1968).

Antioxidant enzymes activities

In this study, the activities of antioxidant enzymes changed differently in response to V. Environmental stresses induce oxidative stress in plants that is evident by high levels of MDA and H_2O_2 (Gopal and Khurana, 2011). Increase in the activities of antioxidant enzymes could be a common strategy for neutralizing the overproduction of ROS (Pandey et al., 2009). However, there are major differences between plants responses. The oxidative damage in plants could be due to H_2O_2 accumulation as a consequence of the enhanced activity of SOD and inhibition of CAT enzymes as that was seen in this study. The respective increase and decrease in SOD and CAT activity in response to metals stress have been reported for the numerous plant species (Pandey et al., 2009). Enhancement in the activity of SOD enzyme in response to vanadium application (at concentration of 40 µM) also was reported for wheat plant under cooper stress

(Wang et al., 2013). Similar to results obtained in this study, the significant reduction in the activity of APX was reported for *Atriplex speciesin* subjected to heavy metals application (Kachout et al., 2009). Glutathione peroxidase (GPX), like APX, detoxifies H_2O_2 to H_2O, but uses GSH directly as the reducing agent instead ascorbate. The regeneration of GSH is made possible by the reduction of GSSG by GR. In this study, substantial reduction was found in the activity of GR. The observed inhibition of GR is parallel to the results obtained by Vestena et al. (2011) to water *Hyacinth* and *Salvinia* in response to cadmium application. In contrast to other H_2O_2 detoxifying enzymes of sunflower APX and CAT and probably GPX, the activity of POD increased significantly in response to V. It could be proposed that POD is responsible for detoxifying the produced molecules of H_2O_2 under vanadium treatment in sunflower. This possibility was supported by the study reported by Gopal and Khurana (2011) on sunflower in response to heavy metals.

Conclusion

In this study, all applied concentrations of vanadium had toxic effects on sunflower plant that was more obvious at the higher concentrations of this element. This toxicity was evident by high production of H_2O_2 and MDA and stunted growth of plant. Sunflower responds to vanadium toxicity by changing some biochemical and physiological features. Accumulation of proline as a compatible osmolytes was a more obvious response of plant to vanadium toxicity. The activities of antioxidant enzymes SOD and POD also increased in response to vanadium existence in root environment. It seems that POD had a significant role in detoxifying the free radicals produced in sunflower plant under the high levels of vanadium toxicity.

Acknowledgment

The authors are grateful to the Research Affairs of University of Payame Noor for financial supports.

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