

## VANADIUM EFFECTS ON PHENOLIC CONTENT AND PHOTOSYNTHETIC PIGMENTS OF SUNFLOWER

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**ABSTRACT.** Vanadium (V) is the 21st most abundant element in the Earth's crust. If some doubt exists about the role of vanadium as a beneficial element, there is no doubt that at high levels it is dangerous to plants. In this study the effect of different concentration of V (0, 3.25, 7.5 and 15 mg L<sup>-1</sup>) was investigated hydroponically in a complete randomized design on sunflower plant. The results indicated that V at all applied concentrations significantly decreased the plant's growth, and total protein and carotenoid contents, but increased the total chlorophyll and soluble sugars contents. Also, the obvious induction in the phenylpropanoid pathway was seen in response to V application. The highest values for PAL (Phenylalanine Ammonia Lyase) activity, and lignin, tannins and flavonoids contents were observed at concentration of 15 mg L<sup>-1</sup> of V. While, the anthocyanin content of plants showed significant increase at concentration of 7.5 mg L<sup>-1</sup> of V. From this study it could be concluded that the applied concentrations of V were toxic for sunflower and the plant by employing the phenylpropanoid pathway efforts to overcome this stress.

**KEY WORDS:** growth, *Helianthus annuus*, PAL, photosynthetic pigments, soluble sugars.

### INTRODUCTION

Vanadium (V) is a transition metal and can exist in a range of oxidation states, from +2 to +5. Vanadium in the form of +5 (vanadate) enters through anionic channels but in the form of +4 (vanadyl cations) come into due to diffusion (Imtiaz et al. 2015). For some mammals such as rats, and green algae V is an essential trace element; however, there is no substantial documents concern with its necessity for humans and higher

plants. Numerous studies have revealed the benefit effects of V on various plant species at lower concentrations (Singh and Wort 1969). However, some other reports have indicated the toxicity of V for other plants species at same quantities (Imtiaz et al. 2015). In most plant species, vanadium toxicity appears at concentrations of 10 to 20 mg L<sup>-1</sup> and causes stunted growth (Vachirapatama et al. 2011), chlorosis (Rosso et al. 2005), disturbed ion uptake (Olness et al. 2001), and elevated reactive oxygen species (ROS) production (Imtiaz et al. 2015). In order to overcome the environmental stresses, some metabolic pathways change, and defense mechanisms including enzymatic and non-enzymatic antioxidant systems activate in plants. Phenylpropanoid pathway, by providing the different metabolites, has a crucial role in plants resistance against unfavorable conditions. The aim of this study was to determine the effects of V on growth and some physiological and biochemical features of sunflower as an important vegetable oil crop worldwide.

## MATERIALS AND METHODS

### Plant growth condition

The seeds of sunflower (*Helianthus annuus* cv. Dorsefid) were germinated in petri-dishes, and then transferred to 50% Hoagland solution for 7 days followed by full strength Hoagland solution. Vanadium as VO<sub>3</sub>NH<sub>4</sub> was applied 7 days after adapting to full strength nutrition at four concentrations (0, 3.25, 7.5 and 15 mg L<sup>-1</sup>). During the seedling growth period, the nutrient solution was replaced every 6 days and pH was set on 5.8 every two days. Photoperiod was 14 hour, light intensity was equivalent to 10 klux and temperature regime was 25/18°C. Plants were harvested 15 days after V treatments, and frozen in liquid nitrogen until assays.

### Photosynthetic pigments assays

Photosynthetic pigments were extracted using 0.1 g of fresh material in 10 mL of 80% aqueous acetone. After filtering, chlorophylls and carotenoids contents were determined spectrophotometrically (Model 1240, Shimadzu, Japan) at 470, 646.8 and 663.2 nm using equations described by Lichtenthaler (1987).

### Total protein and soluble sugars assays

For determination of total protein, 0.1 g of leaf samples were ground at 4°C in 5 mL of phosphate buffer 50 mM (pH=6.8) and then centrifuged at 15000 g for 20 min. To 0.5 mL of supernatant 1.5 mL of Bradford reagent was added and incubated at room temperature for 5 min. The protein content of samples was measured

spectrophotometrically using bovine serum albumin as a standard at 595 nm (Bradford 1976). The soluble sugars were extracted from sunflower leaves ground tissue in 80% ethanol at 4°C for one week. After filtering, to 1 mL of samples 0.5 mL of phenol solution (5%) and 4 mL of sulphuric acid (96%) were added and then incubated at room temperature for 30 min. The absorbance of samples was measured at 490 nm. The soluble sugars content of samples was calculated using glucose as a standard (DuBois et al. 1956).

### Phenolic Assays

Leaf anthocyanins were extracted with methanol: HCl (99: 1). After filtering, the absorbance of samples were measured spectrophotometrically at 550 nm and the anthocyanin content was calculated using an extinction coefficient of  $33000 \text{ M}^{-1} \text{ cm}^{-1}$  (Wanger 1979). Total flavonoids extracted by 80% methanol. An aliquot of extract was mixed with 4 mL of distilled water and 0.3 mL of  $\text{NaNO}_2$  solution (10%). After 5 min, 0.3 mL of  $\text{AlCl}_3$  solution (10%) was added followed by 2 mL of NaOH solution (1%). The absorbance was measured at 510 nm. The total flavonoid content was calculated on the basis of a standard curve of quercetin (Li et al. 2013). Tannin content of samples was determined using insoluble PVPP (polyvinyl-pyrrolidone) as described by Makkar et al. (1993). An aliquot of extract (prepared for total flavonoids) was mixed with 100 mg PVPP, vortexed, left for 15 min at 4°C and then centrifuged for 10 min at 3000 g. In the supernatant the non-tannin phenolics were determined by Folin-Ciocalteu reagent. Tannin content was calculated as a difference between total phenolic and non-tannin phenolic content in the extract. For determining the lignin content dried leaves were homogenized in 99.5% ethanol and the extract was centrifuged at 10000 g for 15 min. The pellet was dried at room temperature. To 10 mg of dried matter 2 mL of acetyl bromide in glacial acetic acid (3.1, V.V) containing perchloric acid (70%, 0.08 mL) was added. After 30 min incubation at 70°C, the samples were cooled with ice and 0.9 mL of 2 M sodium hydroxide and 0.1 mL of 7.5 M hydroxylamine hydrochloride were added. The solution was made up to 10 mL with acetic acid. After centrifugation at 5000 g for 5 min, the absorbance of supernatant was determined at 280 nm and lignin content calculated using the extinction coefficient value of  $20 \text{ g}^{-1} \text{ cm}^{-1}$  (Liyama & Wallis 1988).

### Phenylalanine ammonia lyase (PAL, EC 4.3.1.24) enzyme assay

Leaf samples were homogenized in 50 mM Tris buffer (pH 8.5), containing 1% PVP (polyvinyl-pyrrolidone) and 14 mM 2-mercaptoethanol. The homogenate was centrifuged at 6000 g at 4°C for 10 min. The reaction mixture consisted of 1.9 mL of 50 mM Tris-HCl buffer (pH 8.0), 100  $\mu\text{L}$  of enzyme preparation and 1 mL of 15 mM L-phenylalanine. The assay was started by the addition of enzyme extract after an initial incubation for 60 min at 40°C. The reactions were stopped by the addition of 200  $\mu\text{L}$  of 6 N HCl. The amounts of formed trans-cinnamic acid were determined

by measuring the absorbance at 290 nm. The enzyme activity was calculated using the extinction coefficient of  $9630 \text{ mM}^{-1} \text{ cm}^{-1}$  for trans-cinnamic acid. One unit of activity is defined as the amount of PAL that catalyzes the formation of 1 nM of product at min (Beaudoin-Eagan & Thorpe 1983).

#### Statistical analysis

Assays were carried out in triplicate and the results were presented as mean values  $\pm$  SD. Statistical analyses were performed using a one-way analysis of variance and the significance of the differences between means was determined by Student's multiple range test. The InStat (3.0) software was used to perform statistical analysis.

## RESULTS

### Growth parameters.

In this study, vanadium at all applied concentrations significantly ( $p < 0.05$ ) decreased the fresh and dry masses of roots and shoots (Figures 1-2).

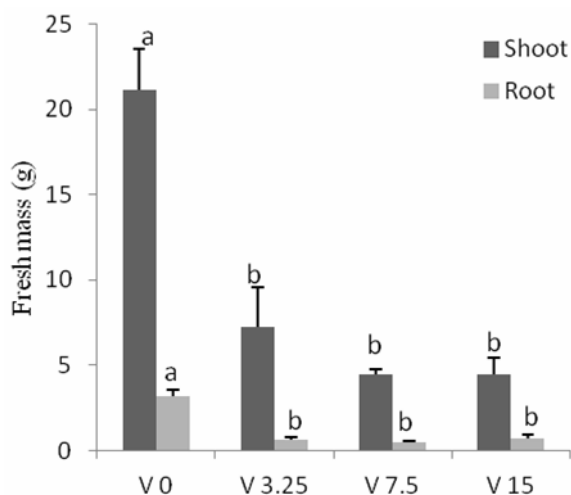


Figure 1. Effects of vanadium on fresh masses of shoot and root in sunflower (V: vanadium).

### Photosynthetic pigments.

Vanadium application at concentration of  $3.25 \text{ mg L}^{-1}$  did not affect the photosynthetic pigments content of sunflower plants, but at concentrations of  $7.5 \text{ mg L}^{-1}$  significantly ( $p < 0.05$ ) increased all chlorophylls contents and

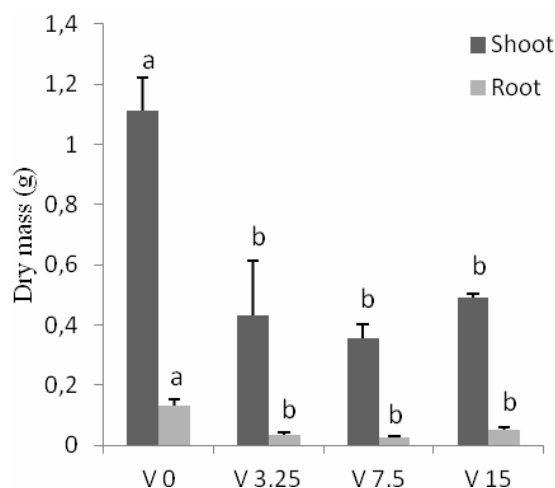


Figure 2. Effects of vanadium on dry masses of shoot and root in sunflower (V: vanadium).

at concentration of 15 mg L<sup>-1</sup> increased the chlorophyll *a* and total chlorophyll contents. On the contrary manner, V application at concentrations of 7.5 and 15 mg L<sup>-1</sup> significantly decreased the carotenoids content of plants (Table 1).

Table 1. Effects of vanadium on photosynthetic pigments and soluble sugars content in sunflower leaves\*.

Treatment	Chlorophyll <i>a</i> (mg g <sup>-1</sup> FW)	Chlorophyll <i>b</i> (mg g <sup>-1</sup> FW)	Total chlorophyll (mg g <sup>-1</sup> FW)	Carotenoid (mg g <sup>-1</sup> FW)	Soluble sugars (mg g <sup>-1</sup> DW)
Control	1.04 ± 0.03 b	0.57 ± 0.04bc	1.62 ± 0.06 b	0.34 ± 0.01 a	12.48±0.41b
V 3.25 mg L <sup>-1</sup>	1.05 ± 0.05 b	0.47 ± 0.06 c	1.48 ± 0.11 bc	0.33 ± 0.007a	13.38±2.44 ab
V 7.5 mg L <sup>-1</sup>	1.26 ± 0.03 a	0.80 ± 0.04 a	2.06 ± 0.07 a	0.24 ± 0.02 b	16.09±1.77 a
V 15 mg L <sup>-1</sup>	1.26 ± 0.02 a	0.72 ± 0.03ab	1.99 ± 0.05 a	0.23 ± 0.01bc	15.85±1.97 a

\*Means followed by the same letter in columns are not significantly different at the 5% level of probability.

### Soluble sugars and total proteins.

According to results, the soluble sugar content of sunflower increased

significantly at concentrations of 7.5 and 15 mg L<sup>-1</sup> of V (Table 1). In contrast, total proteins content of plants decreased significantly in response to vanadium application (Figure 3).

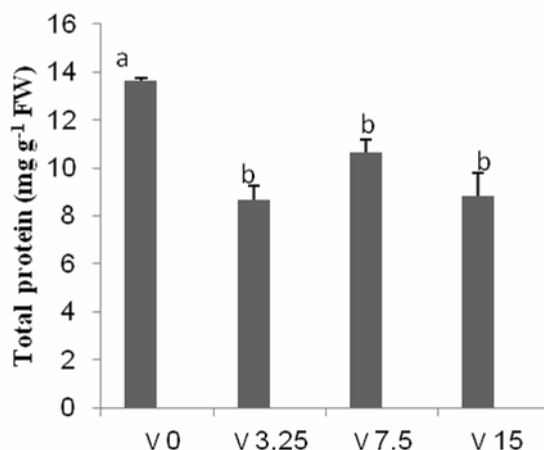


Figure 3. Effects of vanadium on total protein content of leaves in sunflower (V: vanadium).

#### **PAL activity and phenylpropanoid pathway metabolites.**

The activity of PAL enzyme increased significantly in response to V at concentration of 15 mg L<sup>-1</sup> (Figure 4). Vanadium also causes the significant increase in the anthocyanin content of plants at concentration of 7.5 mg L<sup>-1</sup>. In the same way, the total flavonoids, tannins and lignin contents of plant increased by V application. The induced increases in the amounts of total flavonoids and lignin were significant only at concentration of 15 mg L<sup>-1</sup> of V, while tannins increased significantly at both 7.5 and 15 mg L<sup>-1</sup> of V (Table 2).

#### **DISCUSSION**

The stunted growth of sunflower plants that was seen in this study in response to V is similar to results obtained for chinese green mustard and tomato under vanadium treatment (Vachirapatama et al. 2011). The verge value of tolerance to V has been reported to be different among plant species. For example it was reported to be 30 mg L<sup>-1</sup> for soybean (Wang

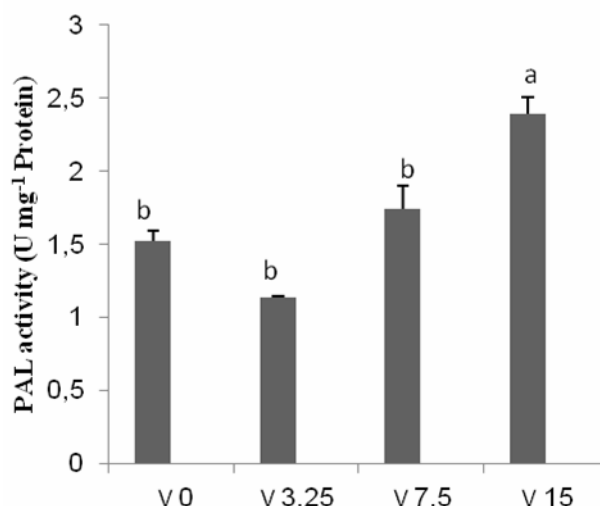


Figure 4. Effects of vanadium on PAL activity of leaves in sunflower (V: vanadium)

Table 2. Effects of vanadium on some phenylpropanoids in sunflower leaves\*.

Treatment	Anthocyanin ( $\mu\text{M g}^{-1}$ FW)	Flavonoids ( $\text{mg g}^{-1}$ DW)	Tannin ( $\text{mg g}^{-1}$ DW)	Lignin ( $\text{mg g}^{-1}$ DW)
Control	$0.57 \pm 0.02$ bc	$14.28 \pm 1.31$ c	$1.11 \pm 0.54$ b	$1.31 \pm 0.08$ b
V 3.25 $\text{mg L}^{-1}$	$0.63 \pm 0.02$ b	$16.33 \pm 0.84$ bc	$0.96 \pm 0.13$ b	$1.62 \pm 0.25$ b
V 7.5 $\text{mg L}^{-1}$	$0.79 \pm 0.02$ a	$15.33 \pm 0.94$ bc	$1.72 \pm 0.1$ a	$2.92 \pm 0.01$ ab
V 15 $\text{mg L}^{-1}$	$0.62 \pm 0.008$ b	$21.53 \pm 0.85$ a	$1.93 \pm 0.59$ a	$3.05 \pm 0.16$ a

\*Means followed by the same letter in columns are not significantly different at the 5% level of probability

and Liu 1999) and  $40 \text{ mg L}^{-1}$  for tomato (Vachirapatama et al. 2011). The antagonistic effect of vanadium with essential nutrients such as Mg, Ca and P has been demonstrated (Olness et al. 2001). Also, the roles of vanadium in induction the stomata closure (Gepstein et al. 1982), ethylene production (Ching 1996) and inhibition the  $\text{H}^+$ -ATPase activity (Imtiaz et al. 2015) also have been reported. These effects are probably the main reasons for the diminished growth of plants under V toxicity. The positive effect of V on chlorophyll biosynthesis that was seen in this study also has been reported

for tomato and maize (Pilbeam 2015). However, there is no justifying evidence concerned with the mechanism of V effect on the chlorophyll biosynthesis in higher plants. According to this study carotenoid content of plants reduced in response to V. Reduction in carotenoid content can be attributed to the changes in the permeability of the membranes and destruction of these components by induced oxidative stress (Kumar et al. 2012). The soluble sugars content of sunflower increased, but the total proteins decreased in response to V. Similar to this, Moreno-Alvarado et al. (2017) reported a significant increase in the soluble sugars content of sunflower in response to aluminum. Carbohydrate accumulation affects the maintenance of cellular membrane and osmotic regulation. Some environmental stresses disturb intracellular water balance and subsequently, change cellular organelles such as tonoplast and enzymes of sugars metabolism. In response to decrease of transferred water to leaves, the content of soluble sugars increases as a mechanism for the maintenance of osmotic potential under stress (Moreno-Alvarado et al. 2017). There is a general consensus that proteins are key targets of heavy metals and metalloids and they can bind to native proteins and inhibit their biological activity (Tamás et al. 2014). The quantitative decreases in the total protein content could be attribute to oxidative stress, modification in gene expression, increased ribonuclease activity, protein utilization by plants for the purposes of metal detoxification and increased hydrolysis of protein that are induced in response to stresses (Alia et al. 2015). It seems that by hydrolysis of soluble proteins, a pool of compatible osmolytes, which are important in osmotic adjustment, is supplied for plants. According to results, the activity of PAL enzyme and studied phenylpropanoids of sunflower increased in response to V. The activation of PAL, a key enzyme in phenylpropanoid pathway, is considered as a common plant response to various stress factors (Nguyen et al. 2016). The potential roles of phenylpropanoids, especially flavonoids, as effective antioxidants have been demonstrated. Flavonoids are also known to enhance plant tolerance against different stress factors such as heavy metals (Izbiańska et al. 2014). Lignin biosynthesis has also been known to be occurred in response to different environmental stresses (Silva Moura et al. 2010). Lignification is presented as a metabolic process that uses ROS in a good manner. Thus lignin participates in an oxidative burst and act as direct protectants against pathogens. Moreover, lignin can act as a stress protectant and offering resistance for stress-challenged plants (Michalak 2006). In overall, phenolic

compounds have hydroxyl and carboxyl groups that able those to chelate metals (Michalak 2006). Phenols are involved in chelating Fe ions and thus, suppressing Fenton's reaction, which is the important source of ROS production. In this study tannin content of sunflower also increased in response to V. It has been reported that plants rich in tannin could tolerate heavy metals by directly chelating the metals (Lavid et al. 2001).

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