The ameliorative effect of silicon and potassium on drought stressed grape (*Vitis vinifera* L.) leaves

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Abstract
The effect of sodium silicate (Si) and potassium (K) were investigated on the major antioxidant enzyme activities in two different grapevine cultivars (*Vitis vinifera* L., cvs Yezandai and Malinger Ramfi) under drought stress. The traits included superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), and also physiological traits such as leaf water content ratio (LWCR), chlorophyll (Chl), soluble protein contents, hydrogen peroxide (H_2O_2), proline (Pro) and glycine betaine (GB) accumulation. The experiment was performed in a completely randomized design including four treatments i.e. the control, drought, Si-drought (0.004 M sodium silicate/kg soil), and K-drought (0.004 M potassium phosphate/kg soil) with three replications in a green house. Drought stress caused a considerable decrease in LWCR, chlorophyll and soluble protein contents. In contrast, compared with the plants treated with drought, applied Si and K significantly enhanced the activities of SOD, CAT, POD, GPX and APX. Under drought stress Pro and GB increased. The results indicated that Si and K offset partially the negative impacts of drought stress by increasing the tolerance of grapevine through rising antioxidant enzyme activities and osmotic adjustment.

Key words: Antioxidant enzymes, Drought stress, Grapevine, Potassium, Silicon.

INTRODUCTION

Abiotic stresses, in particular drought, not only compromise crop quality and limit yield, but also restrict the geographical range over which crop production is viable (Thakur *et al*., 2010). Plant species have evolved a number of physiological and molecular means to cope with adverse environmental conditions (Chen *et al*., 2011). Drought stress inhibits the photosynthesis of plants, causes changes of Chl contents and components and damage to the photosynthetic apparatus (Salekjalali *et al*., 2012). One of the important reasons that environmental stress inhibits the growth and photosynthetic abilities of plants is the breakdown of the balance between the production of reactive oxygen species (ROS) and the antioxidant defense causing accumulation of ROS which induces oxidative stress to proteins, membrane lipids and other cellular components (Shen *et al*., 2010; Tian *et al*., 2013). The antioxidant defense system in the plant cell includes both enzymatic (SOD, CAT, POD, GPX, APX, etc.) and non-enzymatic constituents (Pro and GB, etc.).

Si application shows beneficial effects on plant growth. Although Si is the second most abundant element, it has not yet been listed among the essential elements for higher plants partly because direct evidence that Si is a part of an essential plant constituent or metabolite is lacking (Epstein, 1999). Increasing evidence suggests that improvement of potassium (K)-nutritional status of plants can greatly lower the ROS production by reducing activity of

NADPH oxidases and maintaining photosynthetic electron transport. K deficiency causes severe reduction in photosynthetic CO_2 fixation and impairment in partitioning and utilization of photosynthates (Kakmak, 2005).

Results on the beneficial effects of Si, in enhancing the tolerance to biotic and abiotic stresses, in several plants have been reported. Si benefits to drought tolerance in wheat (Tale-Ahmad and Haddad, 2011), grapevine (Soylemezoglu *et al*., 2009), cucumber (Jiao-jing *et al*., 2009), sorghum (Hattori *et al*., 2005) and salt tolerance (Tripathi *et al*., 2014) have been related to its effect on the antioxidant enzyme activity. Tale-Ahmad and Haddad, (2011) reported that Si alleviates water deficit of wheat by preventing the oxidative membrane damage and may be associated with plant osmotic adjustment. Soylemezoglu *et al.* (2009) reported that Si increase the antioxidant enzymes activity such as CAT in grapevine.

Cakmak (2005) reported that the improvement of K-nutritional status of plants might be of great importance for the survival of crop plants under environmental stress conditions, such as drought, chilling, and high light intensity. In another study Abdel-wahab *et al.* (1995) had reported harmful effects of water deficits can be alleviated by increasing K^+ supplementation in faba bean. Findings of Jonathan *et al.* (2001) had also reported adequate K nutrition can improve drought resistance and root longevity in *Hibiscus rosa-sinensis*.

In this paper, we have investigated the effect of drought stress in two cultivars of grapes, Yezandai and Malinger Ramfi. We also reviewed the effect of Si and K on such grape cultivars in creating drought tolerance.

MATERIAL AND METHODS

Growth conditions and treatments

The research project was conducted at the Faculty of Agriculture, Imam Khomeini International University from April to September 2014. Grape samples were grown as potted vine in the greenhouse with four treatments that included control (C), drought (D), sodium silicate + drought (D-Si), and K + drought (D-K) treatments. Drought stress was imposed by withholding water application. As the grape threshold is -1.0 MPa, therefore the same potential was applied to apply stress. In such conditions soil water potential was –1.0 MPa as measured by gypsum block. The soil structure was a mixture of peat/sand/clay (1:1:1). The soil was mixed sufficiently, divided into several parts, each of 10 kg weight, and then sodium silicate and potassium phosphate (0.004 M of sodium silicate/kg soil and 0.004 M of potassium phosphate/kg soil) were added to D-Si and D-K treatments. Each treatment was replicated three times and the experiment was carried out as a completely randomized design. Leaf samples were collected and frozen in liquid N_2 immediately until analysis.

Measurements of leaf water content ratio (LWCR) and chlorophyll (Chl)

The LWCR of leaves was calculated by the following equation:

$$LWCR = \frac{a-b}{b} \times 100 \quad (1)$$

a: Turgid weight
b: Fresh weight

Leaf Chl was extracted in 80% acetone and the absorbance was read spectrophotometrically at 663 and 645 nm. The Chl content was evaluated using the formula proposed by Arnon (1949).

Measurement of total soluble protein contents

In order to extract total soluble protein, 1 g leaf tissue was homogenized in 3 ml of extraction buffer including 0.5 M Tris-HCl buffer (pH 7.6) and 0.001 M sodium diethyl ditioucarbamate. The homogenate was centrifuged (Beckman Culter, Allegra-64R) at 24 000 × g for 20 min to collect the supernatant as the source of enzyme assays. All the extraction steps were carried out at 4°C. Enzyme activity was estimated spectro-photometrically in laboratory conditions at 25 °C. Total soluble protein was used for the determination of protein content by the method of Bradford (1976). Total soluble protein was also analyzed by SDS-PAGE (12.5% separating and 4.5% stacking gels) at different developmental stages based on the procedure described by Laemmli (1970). The Coomassie Blue staining was used to observe protein bands according to the method of Rybicki and Purves (2003).

Enzyme activity assay

SOD activity was determined by measuring the inhibition in the photochemical reduction of nitrobluetetrazolium at 560 nm as described by Beauchamp and Fridovich (1971). The enzyme activity was expressed as units/mg protein. CAT activity was determined by measuring H_2O_2 consumption at 240 nm for 3 min according to Aebi (1984) method and the enzyme activity was expressed as Δ240/mg protein/min. POD activity was determined by measuring peroxidation of H_2O_2 with guaiacol as an electron donor (Chance and Maehly, 1955). GPX activities were determined by measuring the reduction of guaiacol at 470 nm as described by Urbanek *et al*.

(1991). APX activity was determined following the oxidation of ascorbate to dehydroascorbate, as described by Nakano and Asada (1987) and the enzyme activity was shown as Δ290/mg protein/min. SOD activities were analyzed by Native-PAGE (10% separating and 4% stacking gels). Specified staining of SOD was done according to the method of Beauchamp and Fridovich (1971), CAT and POD activity were estimated on Native-PAGE using 6% separating and 4% stacking gels. POD (EC 1.11.1.7) isoforms were detected according to the method of Hart *et al.* (1971), and finally, to illustrate specified staining of CAT, we used Robertson *et al*. (1987) method.

H_2O_2, Pro and GB determination

H_2O_2 content was determined using the methodology described by Nakano and Asada (1987). Fresh plant material (0.5 g) was homogenized in 5 ml of 1% trichloroacetic acid. The homogenate was centrifuged at 15000 × g for 15 minutes; the supernatant was used for H_2O_2 content determination. Pro was determined according to the method of Bates *et al*. (1973). The amount of GB was estimated according to the method of Grieve and Grattan (1983).

Statistical analysis

The experimental data were analyzed in a completely randomized design with three replicates using software package of SPSS, version 16 and the treatment means were compared by Duncan's multiple range test.

RESULTS

Leaf water content ratio (LWCR)

Compared to the control, the water content of leaves was decreased in Yezandai and M. Ramfi cultivars under drought stress to 19.7 % and 68.6 %, respectively (Figure 1, Table 1). As well, in treatments with Si and K, LWCR increased in Yezandai to 26.6 % and 34.7 %, respectively, compared to the drought stress. Similarly, LWCR also increased in M. Ramfi to 68.73 % and 67.08 %, respectively.

Chlorophyll (Chl)

Chla levels in Yezandai and M. Ramfi under drought stress were decreased to 45.75 % and 49.92 % respectively, compared to the control (Figure 2, A). This reduction in Si and K treatments was 15.12 % and 22.17 % in Yezandai and 10.82 % and 10.52 % in M. Ramfi, respectively. Chl b and total Chl were reduced in Drought treatment (Figure 2, B and C). Application of the Si and K increased the amount of Chl compared to the drought treatment in both cultivars, significantly.

Total soluble protein contents

The amount of total soluble protein in the control and drought treatments showed no significant difference in the Yezandai. However, application of Si and K caused increases in total protein. To analyse changes in total protein content under drought stress, tissue extracts were subjected to SDS-PAGE (Figure 3). Drought stress decreased the total protein compared to the control in both cultivars, significantly.

H_2O_2

The amount of H_2O_2 in leaves under drought stress increased as it was expected. In drought conditions the Si caused decreases in H_2O_2 to 23.95% and 30.67% in Yezandai and M. Ramfi compared to drought without Si. The K treatment also had a positive impact on the reduction of H_2O_2 in both cultivars (Figure 4).

Antioxidant enzyme activity

The activities and electrophoresis pattern of SOD, CAT, POD, GPX and APX, are given in Figures 5 to 9. SOD activity increased to 41.24% and 42.95% under stress, compared to control in Yezandai and M. Ramfi, respectively. Applying Si and K caused these rates to increase to 73.3% and 54.21% in Yezandai and 67.16% and 63.18% in M. Ramfi (Figure 5). CAT activity increased to 68% and 23% under stress, compared to control in Yezandai and M. Ramfi, respectively. Applying the Si and K caused these rates to increase (Figure 6). POD activity in drought stress in both cultivars increased compared to the control, significantly. The POD activity increased to 72.22% and 18.52% in Yezandai and M. Ramfi, respectively. Application of the Si and K improved the POD activity in both cultivars. POD activity in Si treatment had the highest value of 55.56% and 62.96%, in Yezandai and M. Ramfi, respectively (Figure 7). GPX enzyme activity in drought conditions increased to 21.5% compared to the controls in Yezandai cultivar. Si enhanced the activity of this enzyme to 31.58% compared to the control. We did not observe any effects in the GPX enzyme activity after applying the K treatments. The enzyme activity increased to 16% compared to control under drought stress in M. Ramfi. The Si treatment enhanced the activity of this enzyme to 28% compared to the control (Figure 8). APX enzyme activity in the Drought treatment increased to 41.77% and 23.44% in the Yezandai and M. Ramfi, respectively compared to the control. Application of the Si and K treatments caused increases in the activity of APX enzyme dramatically (Figure 9).

Prolin (Pro) and glycine btaine (GB)

Under drought stress Pro was increased to 95.75% and 105.09%, in Yezandai and M. Ramfi, respectively. Treating with Si and K caused more increases in Pro

Table 1. Statistical analysis of different characters for leaves of two grapevine cultivars grown under control (C), Si-drought (D-Si), K-drought (D-K) and drought (D) treatments.

Cultivars	Parameters	F values	C.V. (%)
Yezandai	LWCR (%)	5.89*	10.1
	Chl a (mg/g FW)	16.12**	8.2
	Chl b (mg/g FW)	118.56**	7.6
	Total Chl (mg/g FW)	123.9**	6.3
	Soluble protein (mg/g FW)	4.123*	8.8
	H_2O_2 (µM/g FW)	55.89**	9.1
	SOD (unit/mg protein)	12.12**	8.4
	CAT (µM H_2O_2 dec/min/mg protein)	23.61**	9.9
	POD (µM H_2O_2 dec/min/mg protein)	12.16**	8.6
	GPX (µM H_2O_2 dec/min/mg protein)	4.72*	11.4
	APX (µM H_2O_2 dec/min/mg protein)	53.53**	6.6
	Pro (µM/g FW)	1862.32**	4.3
	GB (µM/g FW)	160.7**	5.6
M. Ramfi	LWCR (%)	6.09*	7.2
	Chl a (mg/g FW)	119.3**	9.3
	Chl b (mg/g FW)	34.84**	7.1
	Total Chl (mg/g FW)	50.29**	9.1
	Soluble protein (mg/g FW)	5.85*	4.2
	H_2O_2 (µM/g FW)	9.134**	8.4
	SOD (unit/mg protein)	7.65**	8.6
	CAT (µM H_2O_2 dec/min/mg protein)	11.95**	11.1
	POD (µM H_2O_2 dec/min/mg protein)	11.95**	8.6
	GPX (µM H_2O_2 dec/min/mg protein)	16.67**	12.3
	APX (µM H_2O_2 dec/min/mg protein)	34.29**	5.8
	Pro (µM/g FW)	684.9**	6.7
	GB (µM/g FW)	271.8**	6.5

The symbols of * and ** in each row represent significant differences at $P < 0.05$ and $P < 0.01$, respectively. Abbreviations are described as follow: LWCR: Leaf water content ratio, Chl a: Chlorophyll a, Chl b: Chlorophyll b, Total Chl: Total Chlorophyll, SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase, GPX: Guaiacol peroxidase, APX: Ascorbate peroxidase, Pro: Proline, GB: Glycine betaine.

content of both cultivars (Figure 10). The GB was increased in drought conditions to 76.16% in Yezandai and 19.4% in M. Ramfi compared to control. The Si increased GB content to 21.5% in Yezandai and 114.81% in M. Ramfi compared to drought without the Si, significantly. The GB content in K treatment also increased to 8.94% and 93.93% in Yezandai and M. Ramfi compared to drought without K (Figure 11).

DISCUSSION

Drought conditions in most plants causes reduced leaf water content (Soylemezoglu *et al*., 2009; Tale-Ahmad and Haddad, 2011). The water content of leaves under drought stress was decreased in both cultivars at the same level, although Yezandai maintained more water. Application of the Si and K caused increases in the water content of leaves. The Si in plants tissues is deposited in cell wall apoplast to form silica and tissue integrity (Sang *et al*., 2002). K is important in plants mainly as an osmotic regulator, and 30-50% of older leaf tissue osmotic potential is regulated by K.

The content of photosynthetic pigments was significantly decreased by drought stress, which is in linewith Nahar *et al*. (2015), Wright *et al*. (2009), and Duli *et al*. (2001) findings, while application of Si and K increased photosynthetic pigments. Si application significantly increased photosynthetic rate of rice plants (Chen *et al.,* 2011), and tomato (Cao *et al.,* 2015) under drought stress. Chl content in water stress conditions can be considered as a limiting factor

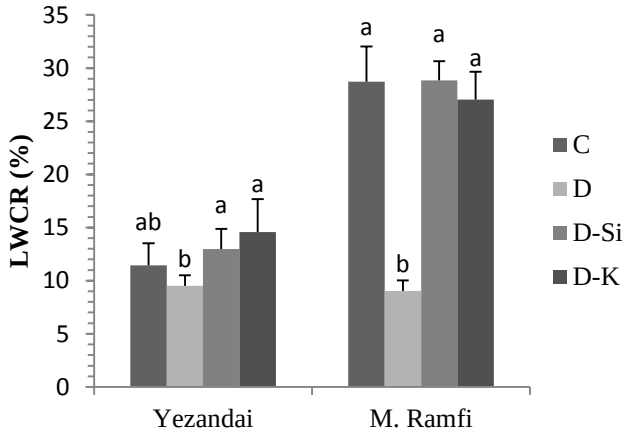

Figure 1. Effect of Si and K on LWCR of two grapevine cultivars under drought stress; data are mean ± S.E. of three replications. Bars with different letters are significantly different at the P < 0.01 level (C: control, D-Si: Si-drought, D-K: K- drought, D: drought treatments).

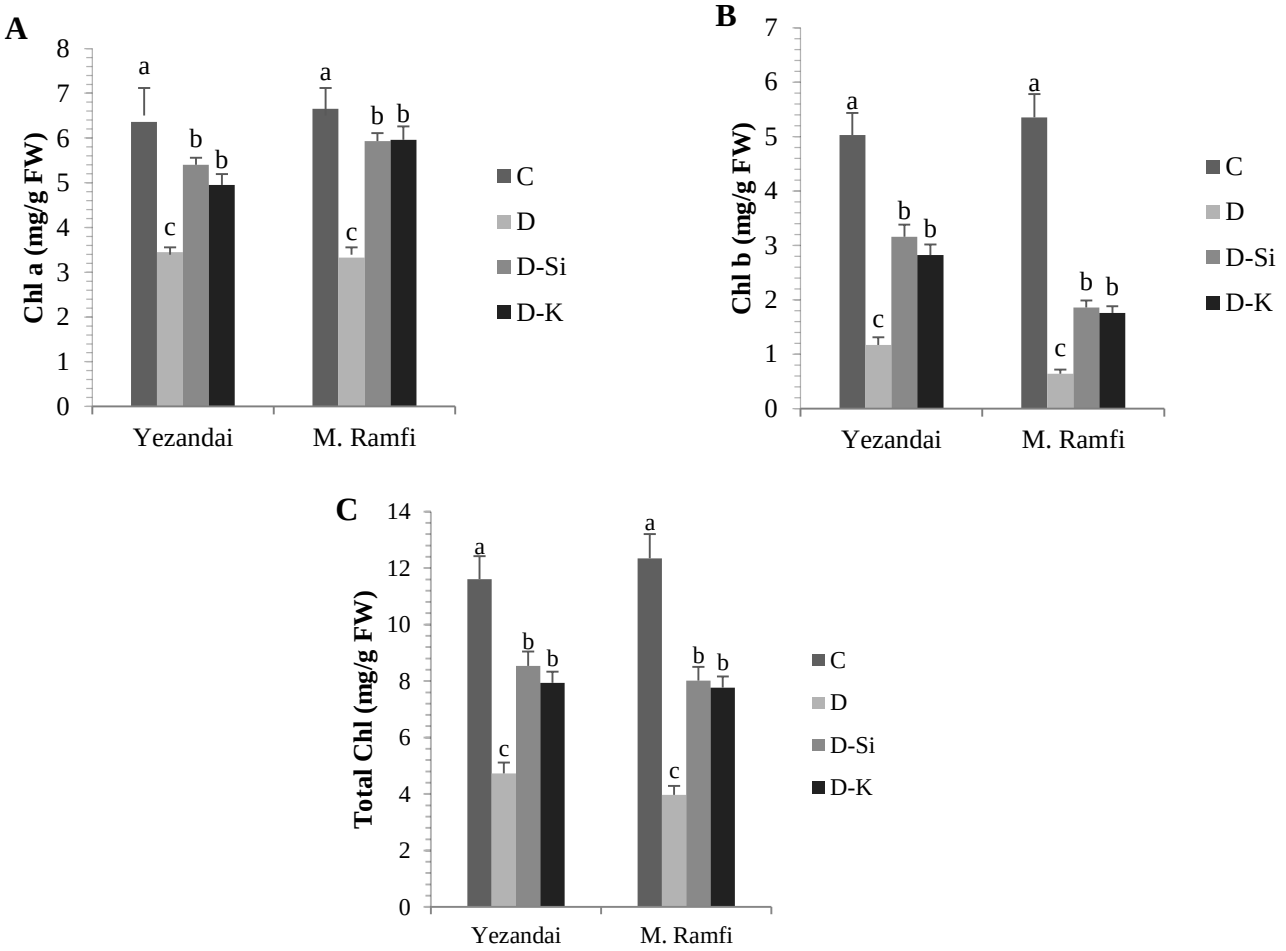

Figure 2. Effect of Si and K on Chl a **(A)**, Chl b **(B)**, and total Chl **(C)** of two grapevine cultivars under drought stress. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P < 0.01 level (The symbols are the same as Figure 1).

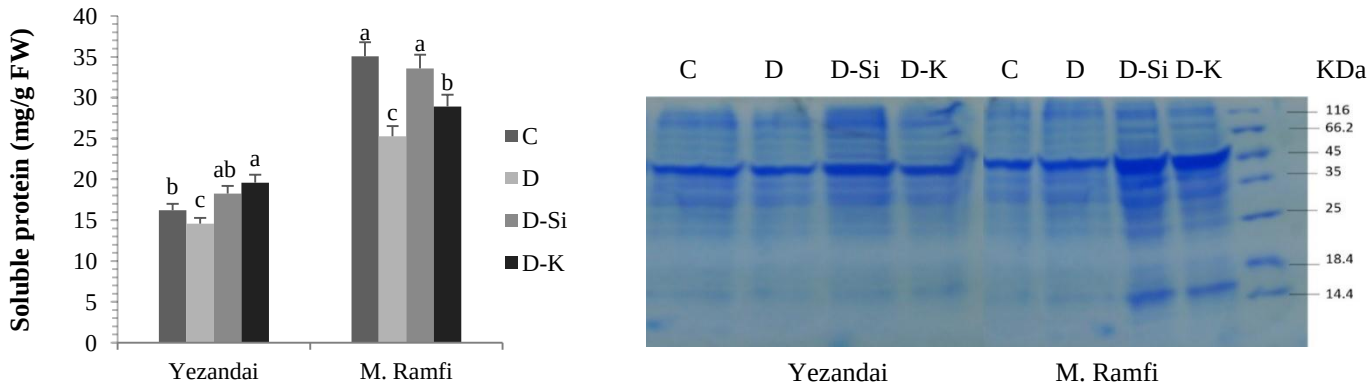

Figure 3. Effect of Si and K on total soluble protein content subjected to SDS-PAGE under drought stress in two cultivars. Bars with different letters are significantly different at the P< 0.05 level (The symbols are the same as Figure 1).

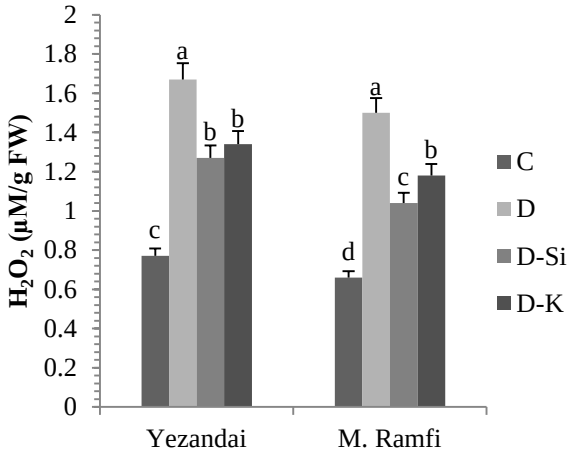

Figure 4. Effect of Si and K on hydrogen peroxide (H_2O_2) content under drought stress in two grapevine cultivars; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

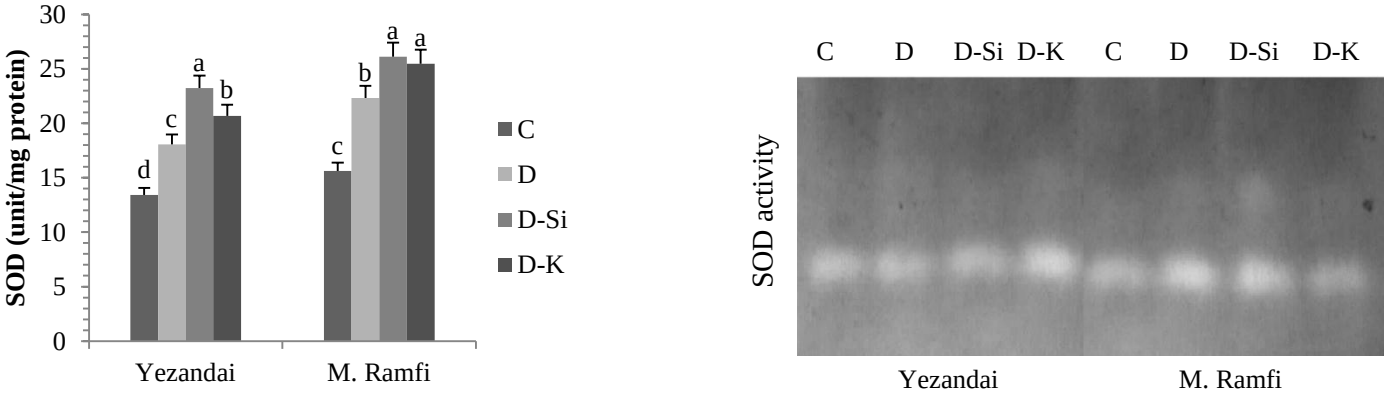

Figure 5. Effect of Si and K on SOD activity and isoenzymes (40 µg protein per well) subjected to Native-PAGE under drought stress in two cultivars. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

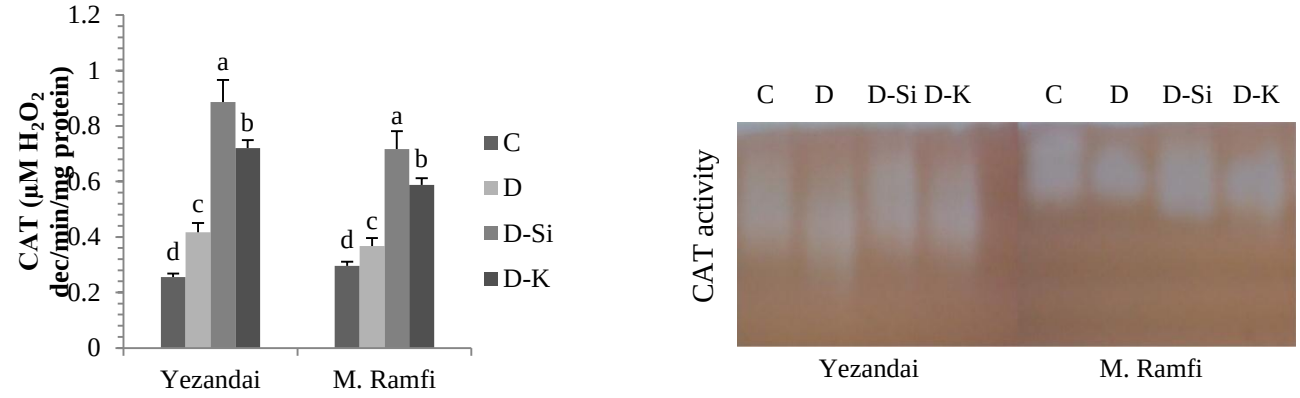

Figure 6. Effect of Si and K on CAT activity (20µg protein per well) subjected to Native-PAGE under drought stress in two cultivars. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

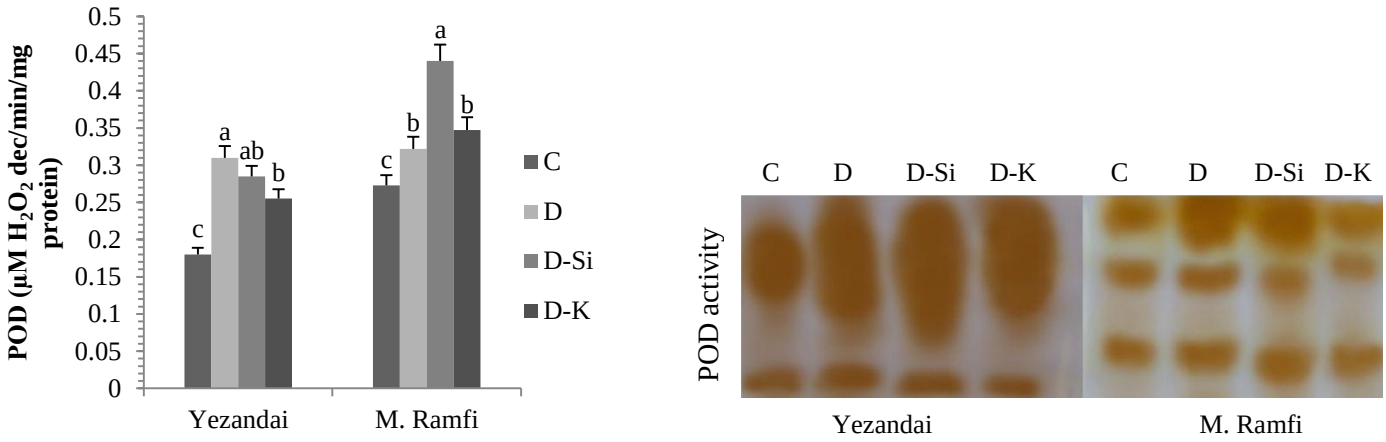

Figure 7. Effect of Si and K on POD activity and isoenzymes (30 µg protein per well) subjected to Native-PAGE under drought stress in two cultivars. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

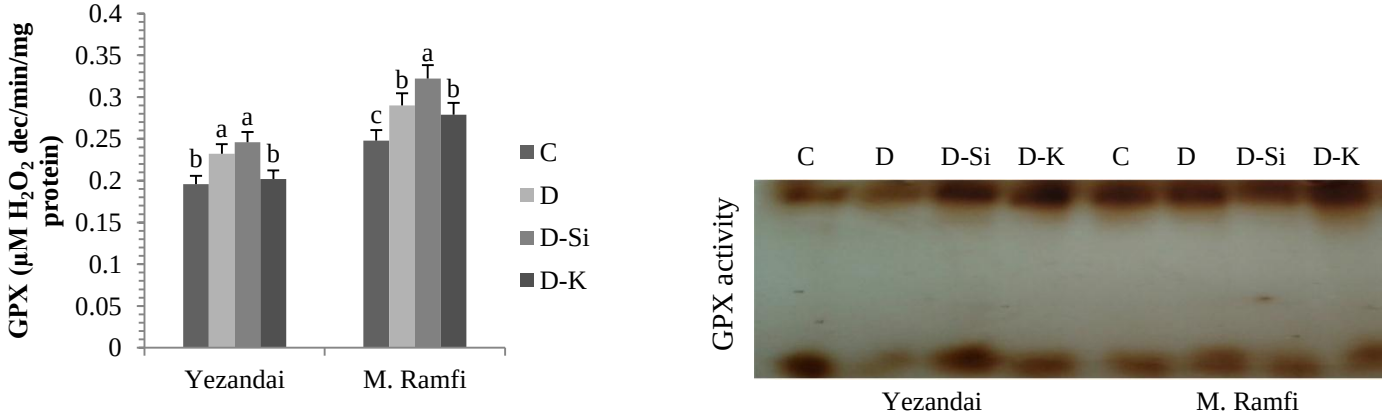

Figure 8. Effect of Si and K on GPX activity and isoenzymes (40 µg protein per well) subjected to Native-PAGE under drought stress in two cultivars. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

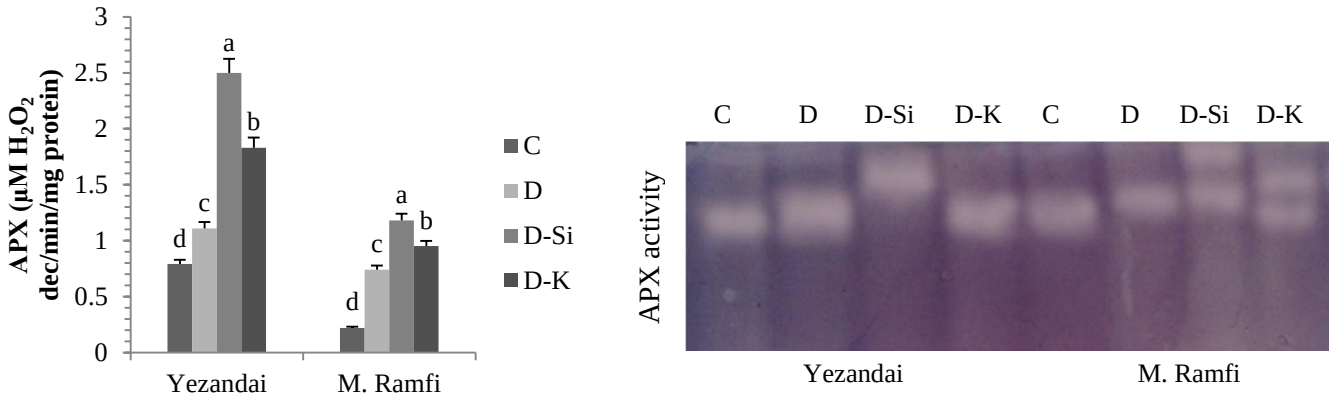

Figure 9. Effect of Si and K on APX activity and isoenzymes (40 µg protein per well) subjected to Native-PAGE under drought stress in two cultivars. Data are mean ± S.E. of three replications; bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

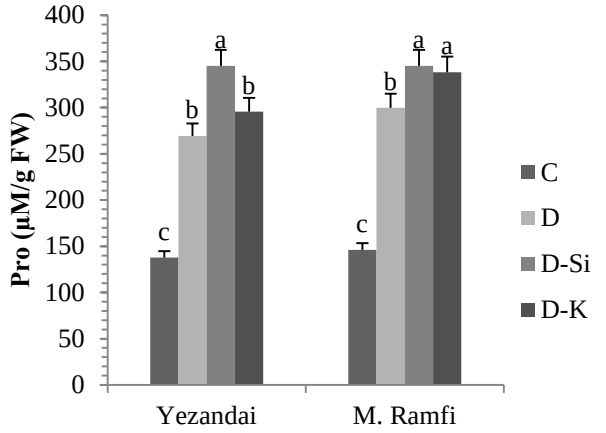

Figure 10. Effect of Si and K on Pro content under drought stress in two grapevine cultivars. Bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

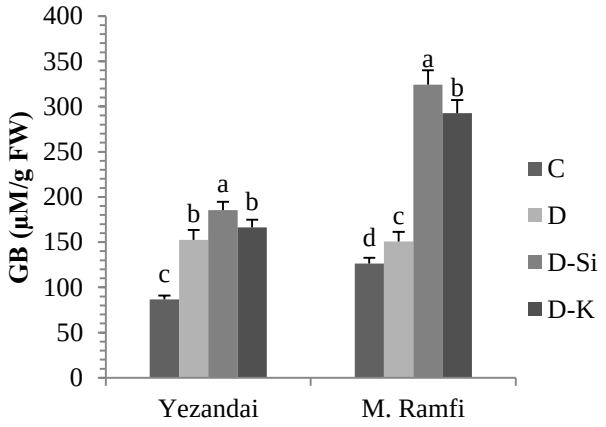

Figure 11. Effect of Si and K on GB content under drought stress in two grapevine cultivars. Bars with different letters are significantly different at the P< 0.01 level (The symbols are the same as Figure 1).

to be considered non-stomatal. One reason for this decline is the increase in chlorophilase enzyme activity that stress induces its expression (Ranjan *et al*., 2001). PS II is very sensitive to light and drought stress, caused by environmental factors in preventing damage to the reaction centers. Under drought stress Chl b was reduced more than Chl a. Oncel *et al.* (2000) reported large amounts of Chl b in chloroplasts, in complex lighting where PS II recipient is located. In these complexes Chl b showed ratio of 3:1, while this ratio is 1:3 in chloroplasts. Researchers mention that in stress conditions, this complex receives more light damage, which causes a drastic reduction in Chl b of chloroplasts.

The tolerance of plants to ROS requires the adaptation of many complex and multifaceted processes. For example, ROS-scavenging enzymes and antioxidant molecules in plants prevent or alleviate the damage from $O_2^{\cdot}$ and H_2O_2 under stress conditions. Superoxide radicals can be dissimulated into H_2O_2 by SOD (Zhang *et al*., 2013) in chloroplasts, mitochondria, the cytoplasm and peroxisomes. Under drought conditions (Gong *et al*., 2005; Cao *et al.*, 2015) and salt stress (Liang *et al*., 2003), the addition of Si increases SOD activity. In our case, based on the results from Native-PAGE, SOD activities were consistent in spectrophotometric data (Figure 5). Si, K and drought increased SOD activity. These results correlate

negatively with $O_2^{\cdot}$ generation. Therefore, an increase in SOD activity can be induced by the addition of Si and K and this will enhance the dismutation of $O_2^{\cdot}$ in drought-stressed grapevine leaves under water deficinecy. H_2O_2 is toxic. To scavenge this molecule, plants have evolved an antioxidant system including CAT, POD, GPX, and APX (Zhang *et al*., 2013). Water deficit stress triggers the activities of CAT, POD and APX (Akitha-Deviand Giridhar, 2015). CAT is the main enzyme that eliminates H_2O_2 in the micro bodies (Shigeoka *et al.,* 2002). In drought stressed grapevine, Si and K 0.004 M/kg-soil increased this enzyme activity significantly (Figure 6). POD as well as CAT, play an essential role in scavenging the H_2O_2 toxicity. GPX may act against the accumulation and toxicity of H_2O_2 in the apoplast (Shigeoka *et al.,* 2002). In our study, drought increased GPX activity and Si led to an even further increase in this enzyme activity (Figure 8). Applying K decreased GPX activity in both cultivars compared to drought, but there were no significant differences between K-D and D stress. Therefore, GPX plays a role in drought-stressed grapevine suffering water deficiency while Si and K reduce lipid peroxidation induced by ROS. The ascorbate-glutathione cycle is found in chloroplasts, and the cytosol (Foyer *et al*., 1994), mitochondria and peroxisomes (Jiménez *et al*., 1997). In this paper, drought condition increased APX activity in both cultivars, and the addition of Si and K further enhanced this activity in drought-stressed grapevine suffering water deficiency (Figure 9). Similar results were obtained by Nahar *et al*. (2015) in *Vigna radiate* L. cv. Binamoog-1. In wheat, Tale-Ahmad and Haddad (2011), in grape, soylemezuglo *et al.* (2009) observed increases in APX activities under salinity stress by Si addition and Zhu *et al.* (2004), also observed increases in SOD and APX activities under salt stress by Si addition in barley and cucumber. This indicates that exogenous Si and K can scavenge H_2O_2 and reduce the lipid per-oxidation via APX.

The addition of Si and K significantly caused increases in LWCR in drought-stressed grapevine leaves that corroborated the increase in Pro and GB concentration (osmotic adjustment). This result was in line with Fariduddin *et al.* (2009) findings. They reported that Pro content exhibited an increase in response to drought stress of leaves in *Brassica juncea*. Moreover, Tale-Ahmad and Haddad (2011) reported Pro and GB contents were significantly increased in Si and drought treatments compared to the control. However, drought stress frequently caused an increase in osmolytes content, so remarkably higher Pro and GB concentrations were observed in Si and K treatments than in other treatments. Pro with osmotic adjustment between cytoplasm and vacuoles and the detoxification of reactive oxygen species cause membrane integrity and stability of the antioxidant enzymes (Ozden *et al.*, 2009). Pro protective mechanism is not yet fully identified, but it plays an important role in reducing the drought damages and also plays a role in macromolecules stability and elimination of free radicals. Pro may remove hydroxyl radicals in the plant and cell acidity as well by reducing the osmotic protective function. Silicon has a positive impact in increasing resistance to environmental stresses related to deposits in the cell walls of roots, leaves, stems and skin. Water deficit causes stomatal closure and reduces the rate of photosynthesis, and silicon reduces stress by decreasing transpiration rate. Basically, leaf transpiration is done through the stomata and the cuticle. Since silicon is deposited on leaf cuticle, a silicon- cuticle makes up the bilayer section in this part of the cuticle and it causes a decreased transpiration rate (Ma and Yamaji, 2006). Silicon and potassium will increase production of Pro and glycine betaine by increasing the osmotic potential, perhaps due to the accumulation of free radicals produced by the plant. Pro and GB are thought to play adaptive roles in mediating osmotic adjustment and protecting sub-cellular structures in stressed plants.

CONCLUSION

The addition of both Si and K caused a significant increase in almost all enzymatic activities, while these caused a decrease in H_2O_2 concentration in drought-stressed grape leaves. Therefore, it might be said that the alleviative effect was attributed to an enhanced antioxidant potential. A significant increase in antioxidative enzyme activities of drought-stressed leaves by both Si and K addition confirmed that these elements could be involved in the metabolic or physiological activity in grape when exposed to drought stress. Generally, it could be concluded that, in grape production, both Si and K are accounted as the essential elements and the presence of sufficient amounts of these two mineral elements help plant survival under drought stress conditions. These results were achieved in the greenhouse under almost controlled conditions and can be difficult to suggest for applying in the field conditions. Such experiments may be organized in the field conditions using Si and K fertilizers. Moreover, doses used were for trial and different amounts of the elements should be tested to achieve the most effective

dose.

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