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Effect of Foliar Application of Methanol on Changes of Antioxidant Enzymes of *Vigna unguiculata* L. in Water-Deficit Stress

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ABSTRACT

The aim of this study was to investigate the effect of foliar application of methanol on the activity of antioxidant enzymes of *Vigna unguiculata* L. in water-deficit stress conditions. The trials were performed in a split plot design arranged in completely randomized block design with three replications in two years cultivation (2017 and 2018). Water-deficit stress treatments were considered in the main plots in four levels (25, 50, 75 and 100% of plant water requirement) and combined foliar application of methanol treatment in three levels (control, 10 and 20% vol.) and genotype treatment in two cultivars of Omidbakhsh 1057 and Mashhad in sub plots. The results showed that the effect of water-deficit stress and foliar application of methanol on the activity of antioxidant enzymes were significant ($P \leq 0.01$). The effect of genotype on the activity of ascorbate peroxidase was just significant ($P \leq 0.01$).

Key words: *Vigna unguiculata* L., Water-Deficit Stress, foliar application of methanol, Antioxidant Enzymes.

INTRODUCTION

Vigna unguiculata L. grows in the tropical and subtropical parts of the world with an annual rainfall range of 300-600 mm (Davoudi *et al.*, 2018). In tropical and subtropical areas, water-deficit stress is a serious threat to the successful production of crops all around the world (Ober, 2001). Water-deficit stress, through the formation of secondary stress such as oxidative stress, may change the synthesis pathways of secondary metabolites (Sharma, 2012). In water-deficit conditions, due to the limited absorption and stabilization of CO_2 and increasing the activity of the oxygenase of the rosiboso enzyme, the respiration increases, which can also increase the production of H_2O_2 (Miller, 2010). The H_2O_2 released to the exterior part of chloroplast has been cleared by the catalase enzyme in leaf cells (Afshar Mohammadian *et al* ., 2016).

Some studies have argued that increasing the CO_2 concentrations could counteract the effects of water-deficit stress (Hsiao, 2000). Therefore, the use of materials that can increase the CO_2 in the plant may improve the plant yield under drought conditions (Zbiec *et al*., 2003). Methanol is a well-known substance for plants, because it is one of the simplest produced compounds, especially during leaf growth and by the pectin demethylation in their cell walls (Haston and Roje, 2001). After the production of this volatile organic compounds inside the plants, some of them release from the leaves and enters the boundary layer, then the atmosphere (Mudgett and Clarke, 1993). Another part turns into aldehyde, then into formic acid and eventually into CO_2. This produced CO_2 can affect the CO_2 assimilation in plants (Galball and Kristine, 2002). Hossinzadeh *et al*. (2015) pointed out that methanol could reduce the water-deficit stress effects with rapid metabolism and increase of CO_2 content with increasing the carboxylation process, reducing the oxygenation and lowering respiration in plants. It can also reduce the amount of hydrogen peroxide in the leaves and finally decreases the activity of peroxidase and catalase enzymes in *Cicer arietinum* L. the present study was conducted to investigate the effect of foliar application of methanol under water-deficit stress conditions on antioxidant enzymes in *Vigna unguiculata* L.

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MATERIALS AND METHODS

In order to investigate the effect of water-deficit stress and foliar application of methanol on antioxidant enzymes of *Vigna unguiculata* L. the field experiments were conducted at Agricultural Research Center of Agriculture and Natural Resources of Safiabad (Dezful, Iran) during the years 2017 and 2018. The experiments were conducted as split factorial based on randomized complete block design with three replications. The studied treatments were water-deficit stress in four levels (25, 50, 75 and 100% of plant water requirement) as main plots and combined methanol with three levels (control, 10 and 20% vol.in the vegetative phase before emerging the flower, twice for ten days) as sub plots and two genotypes (Omidbakhsh 1057 and Mashhad cultivars). The prepared soil was first was passed through a 2 mm sieve, and 1 kg of soil was then poured into each flower pot. To produce water-deficit stress, the flower pots containing 1 kg of soil were placed into an oven at 80°C for 48 h, after which the weight of the dry soil was determined.

The dry soil was poured into another flower pot and water was added slowly up to saturation. The flower pot was then weighed, and the water content was determined by deducting the weight of the flower pot and the dry soil. The various treatments were calculated on this basis. The flower pots were placed in a growth module under a day/night temperature regimen of 25/15°C. Each plot consists of six ridges with a height of 75 cm and a length of 10 m. The plant spacing on the ridge was 20 cm with a row of ridge planting. The distance between the irrigation plots was not doubled and the intervals were 2 m. Planting operations were carried out manually on July 20, according to the custom of the region. After planting, until the establishment of the plant (four leaf), irrigation was carried out normally. Then irrigation was performed based on the treatments.

The modified method of Chang and Kao (1988) was used in order to prepare extraction solution. For extraction of enzymes, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extration procedure was carried out at 4°C. CAT, SOD, APOD and GR were assayed as described previously (Chang and Kao, 1998).

Catalase and guaiac peroxidase (GPX) activity was determined by Chans and Mahley (1955)'s method. For measurement of CAT activity assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Decrease in absorbance of thereaction solution at 240 nm was recorded after every 20 sec. An absorbance change of 0.01 units min^{-1} was defined as one unit CAT activity. Enzyme activities were expressed on protein basis.

The activity of superoxide dismutase was measured according to Giannopolitis and Rice (1977)' method and spectrophotometric method. In this regard, initially, 0.1 gr of the root sample was mixed and uniformed with 3 ml of the reaction solution, including 50 mM potassium phosphate buffer (pH=7.8), 13 mM methionine, 75 µM of tetrazolium, 2 µM riboflavin, 0.1 mM EDTA, and 100 µl of the extraction enzyme. The mixture was placed under bright light conditions of 5,000 lux for 15 minutes. The tetrazolium in the reaction mixture was regenerated under the white light radiation and converted into colored formazan. The maximum absorption of formazan is at the wavelength of 560 nm. On the other hand, the SOD enzyme decreases the amount of tetrazolium regeneration by using H+ in the cell. Accordingly, increasing the concentration of enzyme extract in the regenerative environment of tetrazolium leads to a reduction in the formation of formazan. As a result, the absorption rate decreases at 560 nm (Beauchamp and Fridovich, 1971). Hence, the calibration was increased by adding 1 unit of superoxide enzyme for regenerating 50% of tetrazolium and the activity was recorded the wavelength of 560 nm spectrophotometrically.

Ascorbate peroxidase activity was determined by the Nakano and Asada (1981)'s method. Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm. The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, enzyme and water. The reaction was run for 5 min at 25°C.

The glutathione peroxidase enzyme activity was measured by the Nickel and Cunningham (1969)'s method. The glutathione peroxidase reaction solution consisted of 30 mM $NaPO_4$ buf-fer at pH 6.0, 120 mM H_2O_2,17 mM $Na_2S_2O_3$ and 0.3 mM 2,3',6-trichloroindophenol (Acros, New Jersey). The assay mixture consisted of 950 µl glutathione peroxidase reaction solution and 50 µl of plant extract. The hydrogen peroxide-dependent oxidation of reduced 2,3',6-tri-chloroindophenol was monitored at 675 nm.

Data was analyzed by analysis and variance and mean comparison. Comparison of mean of the treatments was done based on LSD test with SAS software (ver. 9.2).

Results and Discussion

Superoxide dismutase

The results showed that water-deficit stress and foliar application of methanol, as well as the interaction effect of these two treatments on the enzyme activity of superoxide dismutase was significant ($P \leq 0.01$), however, the effect of genotype and the interaction of foliar application of methanol treatments and water-deficit stress and genotype were not significant. The highest enzyme activity of superoxide dismutase was observed in the stress level of 25% of the plant water requirement. On the other hand, among the foliar application of methanol treatments, the results showed the highest activity of this enzyme is related to the treatment without foliar application of methanol (Table 1). In addition, under the interaction effect of water-deficit stress and foliar application of methanol, the highest activity of the superoxide dismutase activity was observed in the stress level of 25% and without spraying (68.8% vol.); however, it was not significantly different from the activity of this enzyme under 10% vol. methanol at the same level of water-deficit stress (0.67) (Table 2). Helal and Samir (2008) found that under water-deficit stress conditions, a significant decrease in superoxide dismutase enzyme activity occurred at flowering stage of *Avena sativa*; however. The reduction in superoxide dismutase activity under water-deficit stress indicated the inhibitory ability of refinement of leaf cells. Gunes *et al*. (2006) attributed the reduction in the activity of this enzyme to the reduction in the synthesis or degradation of this enzyme under stress. One of the reasons of increased activity of superoxide dismutase is the formation of free radicals due to the closure of stomata as a response to drought stress. The closure of stomata due to drought stress reduces CO_2 content in mesophilic leaves and as a result of NADPH accumulation. In this case, oxygen acts as an alternative receptor to electrons, resulting in the formation of superoxide radicals (Yordanov *et al*., 2003). It seems that, after spraying, the methanol converts to CO_2 in the plant and increases the CO_2 content in the cells of the leaf. By performing photosynthesis and further smearing may result in the NADPH consumption produced in the electron transfer chain, thus, its accumulation in leaf chloroplasts and the formation of superoxide is prevented. On the other hand, reduction in the amount of hydrogen peroxide at foliar application of methanol levels of 10 and 20% vol. indicates lower activity of the superoxide dismutase enzyme at these levels.

Catalase

The results showed that the effect of year (P≤ 0.05), foliar application of methanol (P≤0.01) and interaction effect of water-deficit stress and foliar application of methanol was significant on catalase activity (P≤0.01). According to the results of comparison of means, the catalase enzyme level in the first year (0.025) was significantly different from that of the second year (0.024) (Table 1). The highest catalase activity was observed under water-deficit stress of 25% of the plant water requirement, which decreased the activity of this enzyme by decreasing water-deficit stress. In addition, under the influence of foliar application of methanol, the most activity of this enzyme was observed in treatment without spraying (control treatment), which decreased the activity of this enzyme by foliar application of methanol and increasing its concentration (Table 1). Under the interaction of water-deficit stress and foliar application of methanol, the highest activity of this enzyme was observed under water-deficit stress of 50% of the plant water requirement and without foliar application of methanol (control treatment). However, it was not significantly different from the activity of this enzyme under water-deficit stress of 25% of plant water requirement without foliar application of methanol and foliar application of methanol of 10% vol. (Table 2). Ahmed *et al*. (2002) reported catalase enzyme activity in bean plants under water-deficit stress. Since almost 25% of the plant's carbon is consumed for respiration, the foliar application of methanol increases intracellular CO_2 content, which reduces the respiration rate (Ramberg *et al*., 2002). Therefore, by reducing the optical respiration, the hydrogen peroxide produced in the peroxisomes is reduced (Simova-Stoilova *et al*., 2008); as a result, it reduces the amount of hydrogen peroxide and decreases the activity of the catalase enzyme.

Guiacol Peroxidase

Guiacol peroxidase enzyme was also significantly different under the effect of water-deficit stress (P≤0.01), foliar application of methanol (P≤0.01), and interaction effect of

Table 1: Comparison of the means for annual effect of water-deficit stress, foliar application of methanol and genotype on antioxidant enzymes.

Treatment		Superoxide dismutase	Catalase	Guaiacol peroxidase	Ascorbate peroxidase	Glutathione peroxidase
Year	first	0.45^{a}	0.025^{a}	0.029^{a}	0.023^{a}	0.02^{a}
	second	0.43^{a}	0.024^{b}	0.028^{a}	0.022^{a}	0.019^{a}
LSD (0.05)		0	0.0007	0.0009	0.0011	0.0008
Water-deficit Stress	25	0.613^{a}	0.030^{a}	0.041^{a}	0.029^{a}	0.030^{a}
	50	0.396^{a}	0.026^{b}	0.035^{b}	0.022^{b}	0.028^{b}
	75	0.468^{b}	0.023^{c}	0.024^{c}	0.022^{b}	0.013^{c}
	100	0.310^{d}	0.019^{d}	0.15^{d}	0.018^{c}	0.006^{d}
LSD (0.05)		0.019	0.0012	0.0014	0.0014	0.0012
Foliar application of methanol	control	0.499^{a}	0.028^{a}	0.035^{a}	0.025^{a}	0.024^{a}
	10	0.453^{b}	0.025^{b}	0.028^{b}	0.023^{b}	0.023^{b}
	10	0.387^{c}	0.021^{c}	0.023^{c}	0.021^{c}	0.011^{c}
LSD (0.05)		0.0338	0.0009	0.011	0.0007	0.0013
Genotype	Mashhad Cultivar	0.448^{a}	0.025^{a}	0.029^{a}	0.023^{a}	0.020^{a}
	Omidbakhsh 1057	0.445^{a}	0.024^{a}	0.028^{a}	0.022^{a}	0.019^{a}
LSD (0.05)		0.0276	0.0007	0.0009	0.0005	0.0011

The means with similar symbols in each column do not have significant difference.

Table 2: Comparison of means for the interaction effects of water-deficit stress and foliar application of methanol treatments on superoxide dismutase, catalase and ascorbate peroxidase.

Water-Deficit Stress	Foliar application of methanol	Superoxide dismutase	Catalase	Ascorbate peroxidase
25	control	0.68^{a}	0.032^{a}	0.033^{a}
	10	0.67^{a}	0.032^{a}	0.031^{a}
	20	0.47^{b}	0.025^{b}	0.025^{b}
50	control	0.38^{c}	0.033^{a}	0.024^{cb}
	10	0.44^{b}	0.024^{bc}	0.022^{de}
	20	0.36^{c}	0.022^{cde}	0.022^{de}
75	control	0.47^{b}	0.025^{b}	0.023^{cd}
	10	0.46^{b}	0.022^{de}	0.021^{de}
	20	0.46^{b}	0.023^{cd}	0.022^{de}
100	control	0.45^{b}	0.025^{cde}	0.020^{e}
	10	0.23^{d}	0.021^{e}	0.018^{f}
	20	0.24^{d}	0.0160^{f}	0.016^{g}
LSD (0.05)		0.0565	0.0019	0.0015

The means with similar symbols in each column do not have significant difference.

water-deficit stress and foliar application of methanol (P≤0.01), as well as interaction effect of water-deficit stress, foliar application of methanol and genotype. The highest activity of Guaiac Peroxidase enzyme was observed in water-deficit stress of 25% of water requirement and without spray in Omidbakhsh 1057 cultivar, showing a significant difference with other treatments (Table 3). Mercado *et al*. (2004) reported that high activity of guaiac peroxidase was associated with the ability to retain more water in the leaves.

Ascorbate peroxidase: The results showed that the effect of water-deficit stress (P≤0.01), foliar application of methanol (P≤0.01) and the interaction effect of water-deficit stress and foliar application of methanol (P≤0.01), as well as the effect of genotype on activity of ascorbate peroxidase enzyme was significant (Table 4). According to the results of the comparison of means, the maximum activity of ascorbate peroxidase in water-deficit stress of 25% and without foliar application of methanol was observed. However, the activity of this enzyme was not significantly different from that of the stress level and 10% vol. spray (Table 2). The comparison of means also showed that the highest activity of ascorbate peroxidase was observed in Mashhad cultivar (Table 1). The water-deficit stress in three chickpea cultivars significantly increased the activity of ascorbate peroxidase in Arman and Biyonch cultivars (Nasr Esfahani, 2013). Increasing the activity of ascorbate peroxidase enzymes under water-deficit stress is similar to other antioxidant enzymes due to the increase of active oxygen species, which with activating the transcriptional pathways of the message enhances the expression of antioxidant enzymes and increases the activity of these enzymes (Mittler *et al*., 2004). Contrary to the findings of this study, Ozkur *et al.* (2009) showed that there was no significant difference between water-deficit stresses in ascorbate peroxidase activity.

Glutathione peroxidase

The results showed that the effect of drought stress (P≤0.01) and foliar application of methanol (P≤0.01) on glutathione peroxidase activity was significant. In addition, the interaction effect of water-deficit stress and foliar application of methanol (P≤0.01), water-deficit stress and genotype (P≤0.01), foliar application of methanol and genotype (P≤0.01), and water-deficit stress, foliar application of methanol and genotype (P≤0.01) was significant on glutathione peroxidase activity . The results of the comparison of means showed that the highest level of glutathione peroxidase activity was observed for water-deficit stress of 50% of water requirement and 20% vol. foliar application of methanol in Mashhad cultivar (Table 3). According to the findings of the present study, Afkari (2017) reported an increase in glutathione peroxidase activity in basil under water-deficit stress. *Brassica napus* L. leaves showed a significant increase in water-deficit stress in glutathione peroxidase activity (Tohidi Moghadam *et al.*, 2009). The water-deficit stress also increased the glutathione peroxidase activity in sugar beet leaves (Seifzadeh and Rashidi, 2011).

Table 3: Comparison of means for the interaction effects of water-deficit stress, foliar application of methanol, and genotype treatments on guaiacol peroxidase and glutathione peroxidase.

Water-Deficit Stress	Foliar application of methanol	genotype	guaiacol peroxidase	glutathione peroxidase
25	control	Mashhad	0.0415^{b}	0.0102^{gf}
		Omidbakhsh 1057	0.0478^{a}	0.0158^{f}
	10	Mashhad	0.0426^{b}	0.0123^{gh}
		Omidbakhsh 1057	0.0436^{b}	0.0136^{fg}
	20	Mashhad	0.0366^{c}	0.0113^{ghij}
		Omidbakhsh 1057	0.0356^{cd}	0.0123^{gh}
50	control	Mashhad	0.0438^{b}	0.0136^{fg}
		Omidbakhsh 1057	0.0435^{b}	0.0120^{ghi}
	10	Mashhad	0.0336^{de}	0.0105^{ghijk}
		Omidbakhsh 1057	0.0336^{de}	0.0090^{ijkl}
	20	Mashhad	0.0266^{g}	0.0798^{a}
		Omidbakhsh 1057	0.0296^{f}	0.0605^{c}
75	control	Mashhad	0.0350^{cde}	0.0491^{d}
		Omidbakhsh 1057	0.0325^{e}	0.0680^{b}
	10	Mashhad	0.0240^{gh}	0.0120^{ghi}
		Omidbakhsh 1057	0.0230^{hi}	0.0198^{e}
	20	Mashhad	0.0161^{kl}	0.0096^{hijkl}
		Omidbakhsh 1057	0.0188^{jk}	0.0120^{ghi}
100	control	Mashhad	0.0206^{ij}	0.0071^{lm}
		Omidbakhsh 1057	0.0218^{hi}	0.0070^{lm}
	10	Mashhad	0.0126^{mn}	0.0083^{jkl}
		Omidbakhsh 1057	0.0140^{lm}	0.0073^{klm}
	20	Mashhad	0.0115^{mn}	0.0050^{m}
		Omidbakhsh 1057	0.0111^{n}	0.0065^{lm}
	LSD (0.05)		0.0027	0.0033

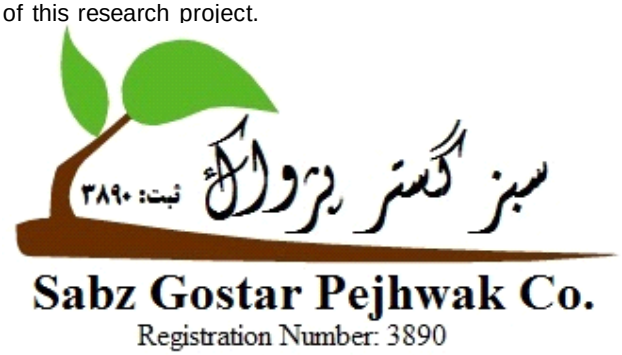
The means with similar symbols in each column do not have significant difference.

Conclusion

Under water-difict stress, plant closes its stomata's to maintain cellular water, thus the photosynthesis reduce due to the lack of CO_2. When the stomata are closed, the optical photosynthesis stage will continue, resulting in the NADPH accumulation. At the same time, oxygen acts as an alternative receptor to electrons, resulting in the formation of superoxide radicals and increasing the activity of antioxidant enzymes under water-deficit stress conditions. Methanol seems to decompose into CO_2 after spraying and absorption by the plant, which results in an increase in the available CO_2 concentration. As a result, CO_2 may continue the process of respiration and the consumption of NADPH obtained from the electron transfer chain (ET_C), which prevent the production of active oxygen species reduce the activity of antioxidant enzymes.

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