Journal of Plant Physiology and Breeding

2020, 10(1): 71-84 ISSN: 2008-5168

Mitigation of drought stress in pot marigold (*Calendula officinalis*) plant by foliar application of methanol

Razieh Khalilzadeh[1*], Raouf Seyed Sharifi[1] and Alireza Pirzad[2]

Received: November 29, 2019 Accepted: June 24, 2020
[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran.
[2]Department of Plant Production and Genetics, Faculty of Agriculture and Natural Resources, Urmia University, Urmia, Iran.
*Corresponding author; E-mail: khalilzadehrazieh@yahoo.com

Abstract
To study the effects of methanol on some physiological and biochemical characteristics of pot marigold (*Calendula officinalis* L.) under drought stress, a factorial experiment was conducted based on a randomized complete block design with three replications under greenhouse conditions in 2017. Factors included the foliar application of methanol at four levels [0 (control), 20%, 30% and 40%] and four irrigation treatments (irrigation at 40, 60, 80 and 100% of field capacity). Increasing water deficit, significantly reduced chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, Fv/Fm and stomatal conductance, whereas 20 and 30% methanol application significantly improved these traits. Irrespective of 40% methanol application, the moderate and severe water deficit treatments led to the decrease of chlorophyll a, total chlorophyll, carotenoid and Fv/Fm. Also, water deficit showed a significant increase in soluble sugars content, proline accumulation and CAT, POD and PPO activity. Compared with the non-methanol treatment, the application of methanol increased the above- mentioned characteristics. The maximum values of these variables were obtained with the application of 40% methanol under severe water limitation conditions. It was indicated that the improvement of biomass due to methanol spraying was associated with the increase of antioxidant defense abilities and maintaining many physiological processes in the stressed plants.

Keywords: Antioxidant enzymes; *Calendula officinalis*; Drought; Methanol; Photosystem II.

Citation: Khalilzadeh R, Seyed Sharifi R and Pirzad A, 2020. Mitigation of drought stress in pot marigold (*Calendula officinalis*) plant by foliar application of methanol. Journal of Plant Physiology and Breeding 10(1): 71-84.

Introduction

Calendula officinalis L. (marigold) from the *Asteraceae* family is an annual herbaceous plant that has been used for herbal medicine, pharmaceutical industry and chemical composition. Flowers of this plant contain compounds that can be broadly applied as an antiseptic, anti-inflammatory and cicatrizing as well as a light antibacterial and antiviral agent (Khalid *et al*. 2010). Changes in physiological and photochemical processes due to a change in environmental conditions such as water deficiency or agricultural practices lead to a change in plant growth and productivity (Pallardy 2010).

Water deficiency imposes one of the most important constraints to photosynthesis, plant growth and crop productivity (Hosseinzadeh *et al*. 2015). The impact of the drought on plant species depends on the variety, severity and duration of the stress as well as on the development stage (Simova-Stoilova *et al.* 2008). Closed stomata which reduce transpiration and conserve water in plants is the first mechanism of plants to face dehydration stress (Sikder *et al*. 2015), which in turn limits carbon fixation, reduces $NADP^+$ regeneration by the Calvin Cycle and decrease the photochemical activities (Arora *et al.* 2002; Monakhova and Chernyadev

2002; Barbosa *et al.* 2015). Exposure of plants to stress is known to induce the formation of reactive oxygen species (ROS), which are involved not only in damage mechanisms but also in cell growth processes (Bernstein *et al.* 2010). ROS such as superoxide, hydrogen peroxide and hydroxyl radical are highly reactive and can seriously disrupt normal metabolism through oxidative damage on lipids, proteins and nucleic acids (Ashraf 2009). Also, water deficit can damage pigments and plastids, reduce chlorophyll *a*, chlorophyll *b* and other carotenoids, hydrolyze proteins and prevalent photochemical reactions in most plants (Reddy *et al.* 2004). Recent investigations have shown that chlorophyll and its derivatives act as antioxidants to prevent oxidative DNA damage and lipid peroxidation both by chelating reactive ions and by scavenging free radicals (Hsu *et al.* 2013). As a result, the induction of antioxidant enzyme activities is a general adaptation strategy that plants use to overcome oxidative stresses (Foyer and Noctor 2003).

The enzymatic antioxidant defense system in the plant cell includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), ascorbate peroxidase (APX), glutathione reductase (GR), etc. Removing the highly toxic H_2O_2 produced during dismutation, is essential for the cell to avoid inhibition of enzymes such as those controlling the Calvin cycle in the chloroplast (Creissen *et al*. 1994), while the H_2O_2 produced can be scavenged by CAT and a variety of POD. CAT, which is only present in peroxisomes, dismutates H_2O_2 into the water and molecular oxygen, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. Different activities of ROS scavenging enzymes including CAT, POD and PPO under stress conditions and at different growth stages have additionally been reported by Khalilzadeh *et al.* (2017), Das and Roychoudhury (2014) and Seysd Sharifi *et al*. (2017). Usually, the amount of the produced dry matter has a direct relationship with the photosynthesis efficiency of the plant and also how CO_2 fixation occurs in crops.

One of the important strategies for increasing carbon dioxide concentration in plants is using chemicals such as methanol that can increase the concentration of CO_2 in a plant and improves photosynthesis rate and growth under water deficit conditions (Ramadant and Omran 2005). Methanol is very simple alcohol, plays a significant role as a precursor in chemical synthesis and/or as a solvent. Additionally, methanol, as the less toxic compound, is used in pharmacology for drug dissolution and the food industry (Pohanka 2016). Several reports suggested that foliar applications of methanol increases CO_2 assimilation in plant's photosynthetic pigments and helps to stabilize photosynthesis under drought stress (Ramirez *et al*. 2006; Ganjeali 2012). Ivanova *et al.* (2001) reported that the foliar application of methanol indirectly stimulates the methyltrophic bacteria that live on most plant leaves. These bacteria consume some of the methanol on the leaves and induce plant growth via auxin and cytokine production. However, the mechanism by which methanol may affect growth and yield is

unknown. It is known that methanol increases stomatal conductance and decreases leaf temperature and transpiration (Makhdum *et al*. 2002), increases glucose metabolism and swelling pressure (Rajala *et al*. 1998), stem length and dry weight (Hernandez *et al*. 2000) and as a result, increases the plant yield. Abundant dioxide carbon supply from methanol causes the photo respiration to be shifted from catabolism to anabolism (Zebic *et al.* 1997). Photorespiration can be minimized with methanol spray, since 25% of carbon wastes during photorespiration (Desclaux *et al*. 2000). That is because methanol is absorbed in the plant and is rapidly metabolized to CO2 in the plant tissue due to the smaller size of methanol rather than CO_2 (Gout *et al*. 2000).

A better understanding of the antioxidant status and physiological responses of pot marigold may help the programs in which the objective is to improve the yield under drought stress. Therefore, this study aimed to evaluate the effects of methanol on physiological responses (i.e., antioxidant enzyme activity, proline, soluble carbohydrate, chlorophyll-a, chlorophyll-b, total chlorophyll, relative water content (RWC), carotenoid, stomatal conductance) of marigold under water stress conditions.

Material and Methods

A factorial experiment based on randomized complete block design with three replications was conducted under greenhouse conditions at Mohaghegh Ardabili University, Iran, in 2017. The area is located at 38° 15′ N latitude and 48° 15′ E longitude with an elevation of 1350 m above mean sea level. Experimental factors included foliar application of methanol at four levels [0 (control) (M_1), 20% (M_2), 30% (M_3) and 40% (M_4)] and four irrigation treatments [irrigation at 40 (I_1), 60 (I_2), 80 (I_3) and 100% (I_4) field capacity]. Pots were filled with a medium that contained one part sand, two parts soil and one part manure. Seeds of marigolds were prepared from Pakan Bazr Isfahan Co. and sown on 21st April in 2017. For each treatment five plants were kept in each pot. Cultural practices such as pest control, irrigation, hoeing and weeding were similar for all treatments. Foliar application of methanol was done at the beginning of flowering (60 days after sowing) in the middle of the day. The plants were watered at the field capacity until the emergence of the second leaf. At this developmental stage, water was withheld to induce water-deficit treatments. The soil humidity was measured when plants had four fully developed leaves (harvest time; the end of the experiment) by weighing soil samples and reweighing them after drying at 105 ◦C for 24 h. Soil humidity was determined at 0-20, 20-40 and beyond 40 cm depth. The measurements were done on five samples for each depth in each water treatment (Sahnoune *et al*. 2004). The soil was silty loam, with the pH of about 6.9. The air temperature of greenhouse ranged from 22-27 °C during the day and 18-21°C during the night. Humidity ranged from 60 to 65%.

The leaves were detached for measuring the following characteristics at the flowering stage. The 1st to 4th youngest leaves were selected as the tissue

samples. The quantum yield and stomatal conductance were measured on the uppermost fully expanded leaf using a fluorometer (chlorophyll fluorometer; Optic Science-OS-30, USA) and leaf porometer (Model SC-J Eijkelkamp, Netherlands), respectively (Kheirizadeh Arough *et al*. 2016). RWC was estimated gravimetrically according to the method of Tambussi *et al.* (2005). Chlorophyll and carotenoids were obtained based on Arnon (1949). Soluble sugars were determined based on the phenol sulphuric acid method (Dubois *et al.* 1956). Leaf proline content was measured according to Bates *et al.* (1973).

To measure the enzyme activity, 0.2 g of fresh tissue was used. To extract the protein, 0.2 g of plant's fresh tissue was crushed by using liquid nitrogen and then 1 ml of buffer Tris-HCl (0.05 M, pH = 7.5) was added. The mixture was centrifuged for 20 min at 16000 ×*g* (13000 rpm) and 4 °C, then the supernatant was used for the enzyme activity measurements. CAT, POD and polyphenol oxidase (PPO) activity was assayed according to Karo and Mishra (1976). To measure the above-ground biomass per plant, five plants of each pot were harvested.

Analysis of variance and comparison of means was performed using SAS computer software. The main effects of factors and their combinations were tested using the least significant difference (LSD) test.

Results

Table 1 showed a significant interaction of water limitation by methanol for the total chlorophyll, chlorophyll-a, carotenoid, Fv/Fm, leaf proline, total soluble carbohydrate, stomatal conductance, biomass, CAT, POD and PPO (Table 1). Chlorophyll-b and leaf RWC were affected by both main factors of water deficit and methanol (Table 1).

Photosynthetic pigment content

The highest chlorophyll-a and total chlorophyll content (8.16 and 13.31 mg g^{-1} FW, respectively) were obtained by the 40% methanol treatment under well-watered conditions (I_1M_4). Whereas, the lowest values (3.12 and 3.99 mg g^{-1} FW respectively) were observed in I_4M_1 (Table 2). Under well-watered conditions (I_1), the chlorophyll-b content was significantly higher than moderate (I_3) and severe drought stress (I_4). Reduction in chlorophyll-b in response to moderate and severe drought stress were 29.32 and 71.58%, respectively. Whereas, plants treated with 40% methanol had significantly higher chlorophyll-b values than the 20% methanol treatment.

Methanol caused increases of total chlorophyll content, from 11.50 mg g^{-1} FW at no ethanol to 13.31 mg g^{-1} FW at 40% methanol under well-watered conditions (Table 2). Increasing methanol concentration tor 30 and 40% decreased chlorophyll-

Table 1. Effects of methanol on the activity of CAT, POD and PPO enzymes, some physiological traits and biomass of pot marigold under water limitation

	Chloryll a (mg g^{-1} FW)	Chlorop hyll b (mg g^{-1} FW)	Total Chlorophyll (mg g^{-1} FW)	Caroten oid (mg g^{-1} FW)	Fv/Fm	Leaf Proline (mg g^{-1} FW)	Total soluble carbohydrate (mg g^{-1} FW)	Leaf relative water content (%)	Stomatal conductance (mmol/m^2/s)	CAT (OD µg protein min^{-1})	POD (OD µg protein min^{-1})	PPO (OD µg protein min^{-1})	Biomass g plant^{-1}
Irrigation (I)													
I_1 = irrigation at 40% FC	7.74a	5.49a	12.61a	1.77a	0.69a	5.05d	167.33d	82.72a	51.52a	0.0067c	0.0106c	0.125d	2.13a
I_2= irrigation at 60% FC	6.50b	4.86a	11.09b	1.32b	0.56b	7.84c	207.26c	68.16b	49.64b	0.0121c	0.011c	0.018c	1.91b
I_3= irrigation at 80% FC	4.65c	3.88b	8.54c	0.89c	0.50c	10.87b	217.38b	64.76c	42.62c	0.027b	0.021b	0.032b	1.79c
I_4= irrigation at 100% FC	3.61d	1.56c	4.92d	0.76c	0.37d	15.18a	228.16a	57.00d	41.52c	0.051a	0.028a	0.043a	1.79c
LSD ($p \leq 0.05$)	0.47	0.36	0.64	0.132	0.05	1.40	7.36	2.28	1.76	0.0097	0.005	0.0037	0.022
Methanol (M)													
M_1= no methanol as control	5.78a	3.43c	8.64b	1.12b	0.46b	8.24b	196.45c	65.89b	44.013b	0.105b	0.011b	0.022d	1.87c
M_2= 20% V	5.72ab	3.64bc	9.36a	1.19ab	0.51ab	8.72ab	202.78b	66.79b	45.51ab	0.024ab	0.017ab	0.025c	1.88c
M_3= 30% V	5.78a	3.82ab	9.55a	1.23a	0.56ab	10.32ab	209.238a	69.57a	47.24ab	0.028a	0.020a	0.028b	1.91b
M_4= 40% V	5.54ab	4.00a	9.608a	1.21a	0.59a	11.65a	211.675a	70.40a	48.37a	0.034a	0.021a	0.030a	1.97a
LSD ($p \leq 0.05$)	0.28	0.36	0.51	0.072	0.104	3.39	4.15	1.64	3.90	0.0165	0.0076	0.0022	0.022
I * M (based on ANOVA)	*	ns	*	**	*	*	*	ns	**	**	**	*	**
C.V.	6.12	11.64	6.59	7.29	4.89	8.50	2.43	2.89	2.08	17.18	10.77	9.74	1.42

ns and *, ** not significant and significant at 0.05, 0.01 probability levels; ANOVA: analysis of variance; CAT: catalase; POD: peroxidase; PPO: polyphenol oxidase.

a, total chlorophyll and carotenoid in the moderate (I_3) and severe (I_4) drought stress conditions as compared with I_1M_0 (Table 2).

Maximum efficiency of PSII (Fv/Fm ratio)

Our findings indicated that I_1M_4 had the higher maximum quantum efficiency of PSII (0.79), whereas I_4M_1 had the lowest maximum quantum efficiency of PSII (0.32) (Table 2). Effect of severe drought stress (I_4) on Fv/Fm ratio was higher than the moderate and low drought stresses (I_3 and I_2) (Table 2).

Proline and soluble sugars

The highest (17.68 mg g^{-1} F_W) and the lowest (4.12 mg g^{-1} F_W) proline content belonged to I_4M_4 and I_1M_1, respectively. Proline content in plants subjected to the moderate drought stress was 22.65 and 30.76% higher than the control when 30% and 40% methanol was applied, respectively (Table 2). Severe drought stress increased the proline content by 32.26 and 44.79% as compared to the control at 30% and 40% methanol, respectively (Table 2). It seems that methanol may improve photosynthesis through an increase in the chlorophyll content (Table 2) which in turn leads to an increase in the amount of assimilates (sugars) produced (Table 2). I_4M_4 and I_4M_3 had higher total soluble carbohydrates than the other treatments.

Stomatal conductance

Our results showed that I_1M_4 had the highest (54.72 mmol/m^2/s) and I_4M_1 had the lowest (40.36 mmol/m^2/s) stomatal conductance among all treatments. Our data also revealed that drought and methanol application treatments significantly differed in terms of stomatal conductance (Table 2). The stomatal conductance was significantly decreased with the increase in drought stress intensity, but with increasing methanol concentration to 40%, stomatal conductance was increased. Plants treated with 30% and 40% methanol increased stomatal conductance at the moderate drought stress as much as 7.43 and 9.41%, respectively. Increased stomatal conductance due to severe drought stress at the above-mentioned methanol concentrations were 2.48 and 4.04%, respectively.

Leaf RWC

Variation of RWC values was observed in response to the drought stress intensity. The adverse effect of water stress on RWC at the severe drought stress was significantly greater than the moderate drought stress (Table 1). RWC decreased about 9.16 and 26.66% in response to moderate and severe drought stresses, respectively as compared to the control (Table 1). The highest RWC (70.40%) belonged to the foliar application of 40% methanol (Table 1) increased it by 6.84% compared to the zero ethanol treatment.

Table 2. The effects of methanol on the activity of CAT, POD and PPO enzymes, some physiological traits and plant biomass of pot marigold under water limitation.

Treatment		Chlorophy	Total	Carotenoid	Fv/Fm	Leaf proline	Total soluble	Stomatal	CAT	POD	PPO	Plant
Water limitation	Bio-fertilizers	ll a (mg g^{-1} FW)	Chlorophyll (mg g^{-1} FW)	(mg g^{-1} FW)		(mg g^{-1} F_W)	carbohydrate (mg g^{-1} F_W)	conductance (mmol/m^2/s)	(OD µg protein min^{-1})	(OD µg protein min^{-1})	(OD µg protein min^{-1})	biomass g $plant^{-1}$
I_1	M_1	7.14±1.42	11.50±2.30	1.54±0.308	0.59±0.11	4.12±0.82	150.36±30.072	48.75±9.75	0.0039±0.0078	0.0060±0.0012	0.008±0.0016	2.11±0.39
	M_2	7.63±1.52	12.52±2.50	1.76±0.352	0.69±0.13	4.05±0.97	162.36±32.47	50.28±10.05	0.0052±0.0010	0.0086±0.0017	0.0095±0.0019	2.12±0.40
	M_3	8.06±1.61	13.11±2.62	1.86±0.372	0.71±0.14	5.13±1.03	172.25±34.45	52.36±10.47	0.0086±0.0017	0.0096±0.0019	0.0158±0.0031	2.15±0.42
	M_4	8.16±1.63	13.31±2.66	1.95±0.390	0.79±0.15	6.91±0.8	184.36±36.87	54.72±10.94	0.0092±0.0018	0.0185±0.0037	0.0160±0.0033	2.16±0.43
I_2	M_1	6.13±1.22	10.36±2.07	1.16±0.232	0.49±0.098	7.16±1.43	198.36±39.67	46.32±9.26	0.0045±0.0009	0.0092±0.0018	0.0111±0.0022	1.91±0.36
	M_2	6.53±1.30	11.09±2.21	1.21±0242	0.52±0.10	6.22±2.39	204.36±40.87	47.82±9.56	0.0086±0.0017	0.0095±0.0019	0.019±0.0039	1.90±0.38
	M_3	7.16±1.43	11.79±2.35	1.58±0.310	0.61±0.12	8.39±1.023	215.96±43.19	51.36±10.27	0.0098±0.0019	0.0125±0.0025	0.020±0.0040	1.92±0.38
	M_4	6.19±1.23	11.15±2.23	1.36±0.270	0.63±0.126	9.60±1.18	210.36±42.072	52.36±10.47	0.025±0.0051	0.0129±0.0025	0.022±0.0044	1.94±0.38
I_3	M_1	5.45±1.09	8.71±1.74	1.02±0.204	0.47±0.094	9.49±1.91	211.45±42.29	40.62±8.12	0.0069±0.0013	0.011±0.0022	0.029±0.0059	1.75±0.35
	M_2	5.15±1.03	9.01±1.80	0.98±0.196	0.49±0.098	9.94±2.015	216.96±43.39	42.36±8.47	0.028±0.0057	0.026±0.0052	0.0301±0.006	1.76±0.35
	M_3	4.16±0.83	8.32±1.66	0.87±0.174	0.54±0.108	11.64±1.72	219.78±43.95	43.63±80.72	0.036±0.0073	0.029±0.0059	0.0325±0.0065	1.80±0.36
	M_4	3.86±0.77	8.12±1.62	0.69±0.138	0.52±0.104	12.41±2.27	221.36±44.27	44.16±8.83	0.036±0.0073	0.018±0.0037	0.0395±0.0079	1.86±0.37
I_4	M_1	3.12±0.62	3.99±0.79	0.77±0.154	0.32±0.064	12.21±2.70	225.63±45.12	40.36±8.07	0.026±0.0053	0.02110±0.0042	0.041±0.0082	1.71±0.34
	M_2	3.58±0.71	4.84±0.96	0.81±0.162	0.34±0.068	14.68±2.94	227.45±5.49	41.58±8.31	0.056±0.011	0.0240±0.0049	0.0425±0.0085	1.75±0.35
	M_3	3.98±0.79	5.64±1.12	0.83±0.166	0.43±0.086	16.15±3.03	228.96±45.79	41.63±8.32	0.058±0.011	0.0320±0.0064	0.0449±0.0089	1.79±0.35
	M_4	3.76±0.75	5.21±1.042	0.65±0.130	0.41±0.082	17.68±2.34	230.62±46.12	42.25±8.45	0.065±0.013	0.0380±0.0076	0.045±0.009	1.93±0.38
$LSD_{0.05}$		0.574	1.021	0.144	0.043	1.38	8.31	1.60	0.007	0.0032	0.0044	0.045

I_1, I_2, I_3 and I_4 indicate irrigation at 40, 60, 80 and 100% field capacity, respectively;

M_1, M_2, M_3 and M_4 indicate the foliar application of methanol at 0, 20% V, 30% V and 40% V, respectively.

The activity of CAT, POD and PPO enzymes

The highest increase in the CAT activity (0.065 OD µg protein min^{-1}) was obtained at the severe drought stress plus 40% methanol (I_4M_4). The 10% methanol under well water irrigation (I_1M_1) had the lowest CAT activity (0.0039 OD µg protein min^{-1}). On the other hand, there was an increase of about 150% and 80.09% in the activity of CAT and POD enzymes, respectively, at the severe drought stress plus 40% methanol (I_4M_4) in comparison with I_4M_1 (Table 2). Increasing the intensity of drought stress led to the increased POD and PPO activity (Table 2). Maximal activities of POD (0.038 OD µg protein min^{-1}) and PPO (0.045 OD µg protein min^{-1}) activity in pot marigold plants were observed under severe drought stress plus 40% methanol (I_4M_4), while the minimum value was observed at I_1M_1 (Table 2).

Biomass

In the methanol-treated plants, the highest biomass (2.16 g $plant^{-1}$) was obtained under well water irrigation plus 40% methanol (I_1M_4), whereas the lowest biomass was obtained at the severe drought stress when no methanol a 12.9% increase in biomass under severe water stress (Table 2).

Discussion

Reduction in chlorophyll concentration is identified as a drought response mechanism to minimize the light absorption by chloroplasts (Pastenes *et al*. 2005). The decreased level of chlorophyll content is caused by photoinhibition and photodestruction of pigments and pigment–protein complexes and destabilization of photosynthetic membrane both induced by drought (Huseynova 2012). Methanol increases stomatal conductance, proline and soluble carbohydrate, cell swelling and chlorophyll and carotenoid content (Zbiec *et al*. 2003; Ramberg *et al*. 2002) which was corresponded with our results. Also, the foliar application of methanol increased photosynthetic capacity and dry matter. Methanol is smaller than the CO_2 and can be easily used by C_3 plants to increase dry matter and as a carbon source (Ramirez *et al*. 2006). Ahmadpour and Hosseinzadeh (2016) reported that increasing the methanol from 25% to 35% decreased chlorophyll content, probably due to the toxic effects of methanol at high concentrations. Since carotenoids play an important role in photoprotection (Munne-Bosch and Penuelas 2003), their increased content under the control (M_1) and the lowest concentration of methanol (M_2), indicates a higher need for photoprotection.

The Fv/Fm ratio is a suitable index for evaluating photosynthetic apparatus in plants exposed to environmental stress (Giorio 2011). Decreasing the Fv/Fm ratio is a reason for the significant effect of environmental stresses on photosynthetic efficiency caused by a decline in the transfer of electrons from PSII to PSI and light protection (Sikder *et al.* 2015). These findings indicate the disorganization of PSII reaction centers under water-stress conditions (Huseynova 2012). Our results showed that the application of the methanol reduced the negative effects of water shortage stress in the pot marigold plants. It is interesting to note that plant growth occurred in

plants that were treated exclusively with methanol. In I_1 and I_2 irrigation levels, increasing methanol concentration led to a gradual increase of PSII efficiency. But at the moderate and severe drought stress (I_3 and I_4), increasing of methanol concentration after 30%, Fv/Fm ratio decreased. Because higher levels of methanol damage chlorophyll and PSII reaction centers, this can happen in drought, heat and light stress conditions (Yazdi Far *et al.* 2015). Therefore, in the present drought conditions, the improvement of photosynthesis of wheat plants under methanol application as compared with the non-methanol treatment was associated with non-stomatal factors. The results of foliar applications of methanol solution under controlled and low-stressed conditions in this study confirm prior observations reporting an increase of growth and yield in plants (Nonomura and Benson 1992). Nonimura and Benson (1992) showed that treated plants with methanol can increase net photosynthesis and improve their performance.

Our results revealed that the accumulation of proline was higher under drought and methanol treatments (Table 2). The extent of these changes was related to the intensity of the stress and concentration of methanol. This phenomenon may be part of a mechanism that prevents loss of water in the plant through osmotic adjustment. The increment of this solute coincided with low rates of maximum efficiency of PSII response to drought conditions (Table 2). In agreement with our results, others have shown that chlorophyll-a and chlorophyll -b positively correlated with RWC and stomatal conductance, but negatively correlated with carotenoid and proline contents (Ghobadi *et al*. 2013). Also, the lower concentration of proline during water deficit was associated with a decrease of stomatal conductance (Pompelli *et al.* 2010). Increased proline accumulation was reported in wheat under drought and salinity stress (Khalilzadeh *et al*. 2017; Seyed Sharifi *et al*. 2017). Proline accumulation under stressed conditions supplies energy for growth and survival and thereby helps the plant to tolerate the stress. The reduced proline oxidase may be the reason for increasing proline accumulation (Manivannan *et al*. 2008). However, in most cases, the osmotic adjustment was not the main consequence of proline accumulation, which was involved in other mechanisms related to sugar content and protection against oxidative damage (de Campos *et al.* 2011). Plants facilitate the decrease of osmotic potential and a further increase of water absorption through an increase in the soluble sugars content. Accumulation of sugars in different parts of the plants has been reported in response to environmental stresses (Prado *et al*. 2000; Khalilzadeh *et al*. 2017).

Foliar applications of methanol may also be used as an appropriate way to enhance the assimilation of CO_2 (Ganjeali, 2012). The positive impact of methanol, however, may be due to its role in reducing photorespiration and enhancing the net photosynthesis process (Nadali 2009). Since the accumulation of carbohydrates has been reported during various abiotic stresses, a decrease in sugar content as a result of the elimination of stress seems reasonable (Archbold 1940).

Foliar applications of methanol under water deficit conditions therefore may alleviate the damages caused by drought stress and also reduce the rate of stomatal conductance in plants. Our results agree with the data obtained by Khalilzadeh *et al.* (2017) and Boureima *et al.* (2012) about the decline in the stomatal conductance in plants subjected to drought stress. Makhdum *et al.* (2002) also reported that foliar application of methanol increased leaf thickness in cotton and this, in turn, led the plants to better maintain RWC in their leaves.

The mechanism of drought tolerance in general, and the mechanism of antioxidant production in particular, differ among species and even among cultivars of a single species. Furthermore, the form and functions of organs and tissue undergo substantial time course changes, so the capability of plants to respond to drought stress depends predominantly on the genes that are expressed at the stage of development during which the stress is imposed (Ashraf 2009).

CAT activity at all methanol concentrations was higher than the non-treated plants. The increase of CAT activity in plants under water stress was also reported in other studies (Quartacci *et al.* 1995; Khalilzadeh *et al.* 2017). Any increase in the activity of antioxidant enzymes may also be associated with the induction of antioxidant reactions which protect plants against oxidative damages. Increasing methanol concentrations increased POD activity under well-water and drought stress conditions. An increase in POD activity was also observed by different authors under drought and saline conditions (Khalilzadeh *et al.* 2012; Kheirizadeh *et al.* 2016; Babaei *et al.* 2017). Increased antioxidant enzyme activities due to the foliar applications of methanol may somehow indicate the alleviation of oxidative stress and the scavenging of ROS by antioxidant enzymes. Preventing the oxidative damages brought to the plant cells during drought stress has been proposed as one of the stress tolerance mechanisms and the extent of this prevention is associated with the increased antioxidant activity (Kalantar Ahmadi *et al.* 2015). Saruhan *et al.* (2012) stated that external application of the growth regulator increased antioxidant enzyme activity in the drought-tolerant maize genotypes compared to the susceptible entries.

Increasing foliar application rates of methanol under normal and stress conditions in the current study increased the pod and PPO activity under drought stress conditions. Stress tolerance in plants may be associated with their ability to scavenge ROS (Saruhan *et al.* 2012). According to the results obtained in the current experiment, however, the effect of methanol in alleviating the negative impacts caused by drought stress was mainly due to an increase in several enzyme activities.

Conclusions

The results showed that water stress reduced the biomass of the pot marigold plants. Methanol foliar application improved total chlorophyll, proline, soluble sugars, stomatal conductance, CAT, POD and PPO enzyme activity under normal and stress conditions. The application of 40% methanol was more effective than the other concentrations. Increasing the methanol from 30% to 40% under drought stress conditions decreased chlorophyll a,

total chlorophyll, carotenoid and Fv/Fm, probably due to the toxic effects of methanol at high concentrations. It seems that improvement of biomass by the application of methanol in pot marigold plants was associated with the increase of antioxidant defense abilities and maintenance of many physiological processes.

Acknowledgements

We are thankful to "National Elites Foundation" for their help during experimentation.

Conflict of Interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

References

Ahmadpour R and Hosseinzadeh SR, 2017. Change in growth and photosynthetic parameters of lentil *(Lens culinaris Medik.)* in response to methanol foliar application and drought stress. International Journal of Agriculture and Biosciences 6(1): 7-12.

Archbold H, 1940. Fructosans in the monocotyledons. A review. New Phytologist 39: 185-219.

Arnon DI, 1949. Copper enzymes in isolated chloroplast polyphenol oxidase in *Beta vulgaris.* Plant Physiology. 24(1): 1-15.

Arora A, Sairam RK and Sriuastava GC, 2002. Oxidative stress and antioxidative system in plants. Current Science 82: 1227-1238.

Ashraf M, 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnology Advances 27: 84-93.

Babaei K, Sharifi RS, Pirzad A and Khalilzadeh R, 2017. Effects of bio fertilizer and nano Zn-Fe oxide on physiological traits, antioxidant enzymes activity and yield of wheat (*Triticum aestivum* L.) under salinity stress. Journal of Plant Interaction 12: 381-389.

Barbosa AM, Guidorizi KA, Catuchi TA, Marques TA, Ribeiro RV and Souza GM, 2015. Biomass and bioenergy partitioning of sugarcane plants under water deficit. Acta Physiologiae Plantarum 37(142): 1-8.

Bates L, Waldren SRP and Teare ID, 1973. Rapid determination of free proline for water stress studies. Plant and Soil 39: 205-207.

Bernstein N, Shoresh M, XuY and Huang B. 2010. Involvement of the plant antioxidative response in the differential growth sensitivity to salinity of leaves vs. roots during cell development. Free Radical Biology and Medicine 49: 1161-1171.

Boureima S, Oukarroum A, Diouf M, Cisse N and van Damme P. 2012. Screening for drought tolerance in mutant germplasm of sesame (*Sesamum indicum*) probing by chlorophyll a fluorescence. Environmental and Experimental Botany 81: 37-43.

Creissen GP, Edwards AE and Mullineaux PM, 1994. Glutathione reductase and ascorbate peroxidase. In: Foyer CH and Mullineaux PM (eds). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, USA.

Das K and Roychoudhury A, 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers of Environmental Science 2: 1-13.

De Campos MKF, de Carvalho K, de Souza FS, Marur CJ, Pereira LFP, Filho JCB and Vieira LGE, 2011. Drought tolerance and antioxidant enzymatic activity in transgenic 'Swingle' citrumelo plants overaccumulating proline. Environmental and Experimental Botany 72: 242-250.

Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28: 350-356.

Foyer CH, Lelandais M and Kunert KJ, 1994. Photooxidative stress in plants. Physiologia Plantarum 92: 696-717.

Ganjeali A. 2012. Effects of foliar application of methanol on growth and root characteristics of chickpea (*Cicer arietinum* L.) under drought stress. European Journal of Experimental Biology 2: 1697-1702.

Ghobadi M, Taherabadi S, Ghobadi ME, Mohammadi GR and Jalali-Honarmand S, 2013. Antioxidant capacity, photosynthetic characteristics and water relations of sunflower *(Helianthus annuus L.)* cultivars in response to drought stres*s.* Industrial Crops and Products 50: 29-38.

Giorio P, 2011. Black leaf-clips increased minimum fluorescence emission in clipped leaves exposed to high solar radiation during dark adaptation. Photosynthetica 49: 371-379.

Gout E, Aubert S, Bligny R, Rebeille F, Nonomura AR, Benson A and Douce R, 2000. Metabolism of methanol in plant cells. Carbon-13 nuclear magnetic resonance studies. Plant Physiology 123: 287-296.

Hernandez LF, Pellegrini CN and Malla LM, 2000. Effect of foliar application of methanol on growth and yield of sunflower *(Helianthus annuus L.).* Phyton, International Journal of Experimental Botany 66: 1-8.

Hosseinzadeh SR, Amiri H and Ismaili A, 2015. Effect of vermicompost fertilizer on photosynthetic characteristics of chickpea *(Cicer arietinum* L.*)* under drought stress. Photosynthetica 54(1): 87-92.

Hsu CY, Chao PY, Hu SP and Yang CM. 2013. The antioxidant and free radical scavenging activities of chlorophylls and pheophytins. Food and Nutrition Sciences 4: 1-8.

Huseynova IM, 2012. Photosynthetic characteristics and enzymatic antioxidant capacity of leaves from wheat cultivars exposed to drought. Biochimica et Biophysica Acta 1817: 1516-1523.

Ivanova EG, Doronina NV and Trotsenko YA, 2001. Aerobic methylobacteria are capable of synthesizing auxins. Microbiology 70: 392-397.

Kalantar Ahmadi S, Ebadi A, Daneshian A, Jahanbakhsh S, Siadat SA and Tavakoli H, 2015. Effects of irrigation deficit and application of some growth regulators on defense mechanisms of canola. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(1): 124-130.

Karo M and Mishra D, 1976. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiology 57: 315-319.

Khalid AA and Teixeira de Silva J, 2010. Yield, essential oil and pigment content of *Calendula officinalis* L. flower heads cultivated under salt stress conditions. Scientia Horticulturae 126 (2): 297-305.

Khalilzadeh R, Seyed Sharifi R and Jalilian J, 2016. Antioxidant status and physiological responses of wheat (*Triticum aestivum* L.) to cycocel application and bio fertilizers under water limitation condition. Journal of Plant Interactions 11(1): 130-137.

Kheirizadeh Arough Y, Seyed Sharifi R and Seyed Sharifi R. 2016. Bio fertilizers and zinc effects on some physiological parameters of triticale under water-limitation condition. Journal of Plant Interaction 11: 167-177.

Makhdum MI, Malik MNA, Uddin SH, Ahmad F and Chudhry FI, 2002. Physiological response of cotton to methanol foliar application. Journal of Research in Medical Sciences 13: 37-43.

Manivannan P, Jaleel CA, Somasundaram R and Panneerselvam R, 2008. Osmoregulation and antioxidant metabolism in drought-stressed *Helianthus annuus* under triadimefon drenching. Comptes Rendus Biologies 331: 418-425.

Mittler R, 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7: 405-410.

Monakhova OF and Chernyadev II, 2002. Protective role of kartolin-4 in wheat plants exposed to soil drought. Applied and Environmental Microbiology 38: 373-380.

Munne-Bosch S and Penuelas J, 2003. Photo-and antioxidative protection during summer leaf senescence in *Pistacia lentiscus* L. grown under Mediterranean field conditions. Annals of Botany 92: 385-391.

Nadali I, Paknejad F, Moradi F and Vazan S, 2010. Effects of methanol on sugar beet *(Beta vulgaris).* Australian Journal of Crop Science 26: 95-108.

Nonomura AM and Beson AA, 1992. The path to carbon in photosynthesis: improved crop yields with methanol. Proceedings of the National Academy of Sciences 89: 9794-9798.

Pallardy SG, 2010. Physiology of Woody Plants. 3th Ed. Academic Press.

Pastenes C, Pimentel P and Lillo J, 2005. Leaf movements and photoinhibition in relation to water stress in field-grown beans. Journal of Experimental Botany 56: 425-433.

Pohanka M. 2016. Toxicology and the biological role of methanol and ethanol: Current view. Biomedical Papers 160: 54-63.

Pompelli MF, Barata-Luís R, Vitorino HS, Gonc ER, Rolim EV, Santos MG, Almeida-Cortez JS, Ferreira VM, Lemos EE and Endres L, 2010. Photosynthesis, photoprotection and antioxidant activity of purging nut under drought deficit and recovery. Biomass & Bioenergy 34: 1207-1215.

Prado FE, Boero C, Gallardo M and Gonzalez JA, 2000. Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinoa Willd*. Seeds. Botanical Bulletin- Academia Sinica 41: 27-34.

Quartacci MF, Pinzino C, Sgherri CLM and Navari-Izzo F, 1995. Lipid composition and protein dynamics in thylakoids of two wheat cultivars differently sensitive to drought. Plant Physiology 108: 191-197.

Ramadant T and Omran Y, 2005. The effects of foliar application of methanol on productivity and fruit quality of grapevine cv. flame seedless. Journal of Grapevine Research 44: 11-16.

Ramberg HA, Bradley JSC, Olson JSC, Nishio JN, Markwell J and Osterman JC, 2002. The role of methanol in promoting plant growth: an update. Journal of Plant Biochemistry and Biotechnology 1: 113-126.

Ramirez I, Dorta F, Espinoza V, Jimenez E and Mercado A, 2006. Effects of foliar and root applications of methanol on the growth of Arabidopsis, tobacco and tomato plants. Plant Growth Regulation 25: 30-44.

Reddy AR, Chaitany KV and Vivekanandan M, 2004. Drought induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology 161: 1189-1202.

Sahnoune M, Adda A, Soualem S, Harch MK and Merah O, 2004. Early water-deficit effects on seminal roots morphology in barley. Comptes Rendus Biologies 327(4): 389-398.

Saruhan N, Saglam A and Kadioglu A, 2012. Salicylic acid pretreatment induces drought tolerance and delays leaf rolling by inducing antioxidant systems in maize genotypes. Acta Physiologiae Plantarum 34: 97-106.

Seyed Sharifi R, Khalilzadeh R and Jalilian J, 2017. Effects of biofertilizers and cycocel on some physiological and biochemical traits of wheat *(Triticum aestivum L.)* under salinity stress. Archives of Agronomy and Soil Science 63(3): 308-318.

Sikder S, Foulkes J and West H, 2015. Evaluation of photosynthetic potential of wheat genotypes under drought condition. Photosynthetica 53: 47-54.

Simova-Stoilova L, Demirevska K, Petrova T, Tsenov N and Feller U, 2008. Antioxidative protection in wheat varieties under severe recoverable drought at seedling stage. Plant, Soil and Environment 54: 529-536.

Tambussi EA, Nogués S and Araus JL, 2005. Ear of durum wheat under water stress: water relations and photosynthetic metabolism. Planta 221: 446-458.

Yazdi Far S, Moradi P and Yousefi Rad M, 2015. Effect of foliar application of methanol and chelated zinc on the quantities and qualities yield of marigold *(Calendula officinalis L.).* Journal of Applied Environmental and Biological Sciences 4(12): 170-176.

Zbiec L, Karczmarczyk S and Podsiadlo C, 2003. Response of some cultivated plants to methanol as compared to supplemental irrigation. Electronic Journal of Polish Agricultural Universities 6 (1): 1-7.

تعدیل اثرات تنش خشکی توسط محلوپاشی متانول در گیاه همیشه بهار (*Calendula officinalis* L.)

راضیه خلیل‌زاده[1*]، رئوف سید شریفی[1] و علیرضا پیرزاد[2]

۱- گروه زراعت و اصلاح نباتات، دانشکده کشاورزی و منابع طبیعی، دانشگاه محقق اردبیلی، اردبیل.
۲- گروه تولیدات و ژنتیک گیاهی، دانشکده کشاورزی و منابع طبیعی، دانشگاه ارومیه، ارومیه.
*مسئول مکاتبه: E-mail: khalilzadehrazieh@yahoo.com

چکیده

به منظور بررسی تأثیر متانول بر برخی خصوصیات فیزیولوژیکی و بیوشیمیایی گیاه همیشه بهار (*Calendula officinalis* L.) در شرایط تنش خشکی، یکآزمایش فاکتوریل در قالب طرح بلوک‌های کامل تصادفی با سه تکرار در شرایط گلخانه در سال ۲۰۱۷ انجام شد. استفاده از متانول در چهار سطح (شاهد، ۲۰٪، ۳۰٪ و ۴۰٪) و چهار سطح آبیاری (آبیاری در ۴۰٪، ۶۰٪، ۸۰٪ و ۱۰۰٪ ظرفیت زراعی) را شامل شدند. با کاهش کمبود آب ، کلروفیل a، کلروفیل b، کلروفیل کل، کاروتنوئید، کارایی فتوسنتزی و هدایت روزنه‌ای کاهش یافت، در حالی که کاربرد ۲۰ و ۳۰ درصد متانول به طور معنی‌داری این صفات را بهبود بخشید. صرف نظر از استفاده از متانول ۴۰ ٪، کاهش کمبود آب در حد متوسط و شدید منجر به کاهش کلروفیل a، کلروفیل کل، کاروتنوئید و کارایی فتوسنتزی گردید. کمبود آب در محتوای قندهای محلول، تجمع پرولین و فعالیت CAT، POD و PPO افزایش معنی‌داری نشان داد. در مقایسه با تیمار غیر متانول، استفاده از متانول موجب افزایش متغیرهای ذکر شده گردید. حداکثر مقادیر این متغیرها با استفاده از متانول ۴۰٪ در شرایط محدودیت شدید آب به دست آمد. به نظر می‌رسد که بهبود بیوماس گیاه در اثر محلول‌پاشی متانول با افزایش توان دفاعی آنتی اکسیدانی و نگهداری فرایندهای فیزیولوژیک گیاهان تنش زا همراه است.

واژه‌های کلیدی: آنزیم‌های آنتی اکسیدانی؛ خشکی؛ فتوسیستم ۲؛ متانول؛ *Calendula officinalis*.