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Antioxidant Activity: A Strategy for Alleviating the Effects of Drought on *Calendula officinalis* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Author ME implemented the work, data analysis, sampling, experiments and wrote the manuscript. Author GRZ consulted for the implementation of the work, provided materials and equipments, revised data analysis and wrote the manuscript. Author ZA consulted for doing experimental sections, designed antioxidant and molecular experiments, revised and wrote the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Effects of drought stress on pot marigold and plant's antioxidant responses in confronting with water deficit was studied.
Study Design: The experiment was conducted according complete randomized block design (CRBD) with three replications. Drought stress with four levels (80, 60, 40 and 20% of the soil available water content) and plant type (medicinal and ornamental) were considered. Means were compared using LSD test. Correlation analysis was done using Pearson Correlation Coefficient.
Place and Duration of Study: Research greenhouse of faculty of agriculture, University of Birjand, Iran; Between December 2014 and June 2015.
Methodology: Enzymatic antioxidants activity and non-enzymatic antioxidants content along with MDA (malondialdehyde) content were determined spectrophotometric and flower yield was consisted of the oven dried flowers collected during the reproductive stage.
Results: MDA content increased with increasing drought intensity from 17.97 in well-watered to 28.67 mmol.gr^{-1} fresh weight in severe stress level. SOD and CAT activity primarily increased to

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8.58 u.mg^{-1} protein and 4.48 ΔA240.mg^{-1} protein in moderate stress level respectively and then decreased afterwards. APX activity decreased from 2.37 in well-watered level to 1.31 ΔA290.mg^{-1} protein in severe stress level. Proline content increased accompanied by increasing drought, so that in the highest level (36.17 mg.gr^{-1} fresh weight) was up to fourfold compared with non-stressed control. However carotenoid content concurrently decreased from 4.50 to 3.30 mg.gr^{-1} fresh weight. With increasing drought, flower yield decreased with a certain rate, so the highest (0.52 g.plant^{-1}) and lowest (0.21 g.plant^{-1}) flower yield belonged to non-stressed and intensive drought stress, respectively. In addition, flower yield of medicinal type was approximately 24% higher than ornamental one.

Conclusion: Enzymatic antioxidant system of pot marigold confers a suitable ability to reduce adverse effects of drought-induced oxidative stress. However when the intensity of drought stress is high, this scavenging system fails to cope with higher levels of oxidative compounds.

Keywords: Reactive oxygen species; cell membrane; proline; malondialdehyde.

ABBREVIATIONS

SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; POD: peroxidase; GR: glutathione reductase; Cys: cysteine; GSH: reduced glutathione; Asc: ascorbic acid; Pro: proline; TCP: tocopherls; GP: guaiacol peroxidase; POX: peroxidase; DHAR: dehydroascorbate reductase; MDHAR: monodehydroascorbate reductase; MDA: malondialdehyde; RWC: relative water content; MT: medicinal type; OT: ornamental type; ROS: reactive oxygen species; FC: field capacity; PWP: permanent welting point; AWC: available water content; FW: fresh weight; DW: dry weight; TW: turgid weight; LSD: least significant difference; PEG: polyethylene glycol.

1. INTRODUCTION

One reason for the reduction of growth and photosynthetic ability under environmental stresses such as drought is that the equilibrium between production and removal of the reactive oxygen species is disturbed in such conditions, resulting in the accumulation of these damaging species and the induction of the oxidative stress which could then damage proteins, membrane lipids and other cellular components [1]. Photosystem II reduced activity during the drought stress due to the imbalance between absorbance and usage of the radiation energy in turn disturbs the balance between the production and usage of the electrons [2]. The leaked electrons from these chains could react with oxygen during the normal and abnormal metabolism to produce ROS [3]. To survive under such undesirable growth conditions, plants have developed unique defense mechanisms for acclimation that increase their tolerance to detrimental conditions [4]. One of the defense mechanisms against different stresses is the antioxidant enzymes production. In fact plants protect themselves by increasing scavenging capacity of ROS via antioxidant enzymes and molecules [5]. The antioxidant defense system in the plant cell includes enzymatic and non-enzymatic constituents. Despite there are extensive studies on the effects of environmental stresses on the growth and yield of crops, there is very few information about medicinal herbs responses to these stresses [6].

Besides being an edible ingredient as food flavoring and coloring, the flowers of Pot marigold (*Calendula officinalis* L.) contains active ingredients and components to be used in industry and pharmaceutical industry [7]. Despite the effect of drought stress on some morphological, physiological and yield related traits [8] and antioxidant responses of marigold to other environmental stresses such as salt stress [9] is well studied, based on our knowledge, there is very little information about its antioxidant responses to oxidative stress induced by water deficit [10]. We therefore wanted to study some aspects of plant resistance to drought stress which are related to its antioxidant ability and are not studied to date.

2. MATERIALS AND METHODS

2.1 Plant Materials and Water Regime Implementation

The experiment was conducted as a factorial with the basis of a complete randomized block design in the research greenhouse of Birjand

University. In block designing, in addition to the growth condition, plant size at the beginning of the drought imposing was also taken into account. Therefore, we tried to put plants with the same size, in the same block. There were two factors including drought stress with four levels and plant type with two levels and three replications for each in the experiment. Drought stress levels consisted of moisture retention at 80 (well-watered) 60 (mild stress) 40 (moderate stress) and 20 (severe stress) percent of the soil available water content. Two calendula types including pharmaceutical (sparse petals) and ornamental (bushy petals) made the second factor. To determine the moisture content of the soil field capacity (FC), pressure plate method was employed [11] and permanent wilting point (PWP) was assumed to be half the FC [12]. So the moisture content between FC and permanent welting point (PWP) is soil available water content (AWC). Two types of marigold seeds were purchased from Pakan Bazr Isfahan Company and after disinfection planted directly in square shaped pots filled with 6.75 kilograms of a loamy soil. Daily full watering (watering up to FC) by weighting the pots was done before the drought stress was imposed. Drought stress was imposed by daily weighting and watering the pots to the intended moisture levels, after we made sure there are enough well established plants in each pot. Plants were grown under natural light in a greenhouse with a day/night temperature of 25/18 degrees centigrade, and the relative humidity was approximately 40 percent. Pots were on a metal platform with a height of about 1 meter from the surface. Plants were grown from December 2014 to May 2015.

2.2 Measuring the Studied Traits

When 50% of plants belonged to each treatment started flowering, the last fully expanded leaf of one plant of each plot that had been previously selected randomly was separated. Separated leaves then were immediately put in an aluminum foil and placed in liquid nitrogen tank to be frozen. After taking the tank to the laboratory, leaf samples were completely powdered in Chinese mortar using liquid nitrogen and the required amounts for each measurement (including antioxidant activity, content of chlorophyll pigments, proline content and malondialdehyde (MDA) content) were placed in separate vials. Vials were then kept in a -20℃ freezer.

2.2.1 Determining membrane peroxidation (MDA test)

To determine membrane peroxidation, MDA content was measured as the final product of this reaction and its content was expressed in milimol per gram fresh weight of the used plant frozen tissue [13].

2.2.2 Determining antioxidant enzymes activity

Approximately 200 milligrams of each frozen sample was used to measure antioxidant activity and at the same time, protein content was also determined using the proposed method by Bradford [14]. Leaf samples were taken from the last fully expanded leaf of on plant from each experimental unit.

2.2.2.1 Superoxide dismutase (SOD) activity

SOD activity was determined using photochemical method [15]. Leaf samples (approximately 0.2 g) were homogenized in 3 mL HEPES-KOH buffer (pH 7.8) with 0.1 mM EDTA called extraction mixture. Cooled homogenate was centrifuged at 12,000 rpm for 15 minutes. The supernatant (superficial solution) was used as a source of SOD enzyme. The reaction mixture (3 mL) contained 0.1 mM EDTA, 50 mM HEPES-KOH buffer (pH 7.8), 50 mM Na2CO3 (pH 10.2), 12 mM Lmethionine, 75 NBT, 300 µL enzyme extract and 1 µM riboflavin. The absorbance was read at 560 nm and one unit activity of SOD was defined as the rate of enzyme required to result in a 50% inhibition of rate of NBT reduction.

One unit activity of SOD was defined as the amount of enzyme required to result in a 50% inhibition of rate of nitroblue tetrazolium bromide (NBT) reduction in 560 nm wavelength.

2.2.2.2 Catalase (CAT)

CAT activity was measured by the method proposed by Cakmak and Horst [16]. The extraction mixture and extraction method was the same as SOD. The reaction mixture consisted of 2.6 ml of 25 mM Na-phosphate buffer (pH 6.8), 400 µL of 10 mM H2O2, and 40 µL of enzyme. The decomposition of H2O2 was followed by the decline of absorbance at 240 nm. Enzyme activity was expressed according to absorbance changes relative to milligram protein in the extract.

2.2.2.3 Ascorbate peroxidase (APX)

APX activity was measured by the method of Nakano and Assada [17]. Leaf samples of approximately 0.2 g were homogenized in 1 mL of 50 mM Na-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM ascorbate, 5 mM DTT, 100 Mm NaCl and 2% (w/v) polyvinylpyrrolidone. The mixture was then centrifuged at 12,000 rpm for 15 minutes. The supernatant was used as a source of enzyme. The reaction was initiated by adding H_2O_2 to a solution with final concentration of 44 µM. The decrease in absorbance was monitored at 290 nm. The rate of APX was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and APX activity was expressed as absorbance changes relative to milligram protein in the extract.

2.2.3 Determining nonenzymatic antioxidants

2.2.3.1 Carotenoids

Carotenoid content as an antioxidant agent was determined using Arnon [18] method and its content was reported in gram carotenoid per gram fresh weight of the sample.

2.2.3.2 Proline content

Proline content was measured and reported in milligram per gram fresh weight of the plant tissue using the standard curve [19].

2.2.4 Relative water content (RWC)

RWC was measured by the method proposed by Bars and Weatherly [20] using the following equation:

$$RWC\ (\%) = (FW\text{-}DW)/ (TW\text{-}DW) * 100$$

Where FW is Fresh weight, DW is dry weight and TW is the turgid weight of leaves after soaking in deionized water during the night.

2.2.5 Flower yield

At the beginning of the experiment, 8 plant of each pot were randomly selected and their flowers were daily collated until the end the experiment. Collected flowers were then air-dried in the dark and weighted using a scale with an accuracy of 0.0001 gram. Flower yield consisted of the whole flowers collected during the reproductive stage was expressed in grams per plant.

2.3 Data Analysis

The statistical software SAS (version 8.0 for windows) was used to analyze experimental data and means were compared according to Least Significant Difference (LSD) test. Correlation analysis was done using Sigma Plot (version 11.0) and curves and charts were drawn in Excel 2010. P values less than 0.05 were considered as significant.

3. RESULTS AND DISCUSSION

3.1 Lipid Peroxidation Rate (MDA Content)

Analysis of variance revealed that there was a significant difference between different levels of drought stress, although two plant types were not different in terms of MDA (Table 1). Mean comparison of drought levels showed that, in general, MDA content was increased with increasing drought intensity (Table 2).

These results correspond with those reported by Mirzaee et al. [21] where MDA content increased in harmony with increasing polyethylene glycol (PEG) in canola root and shoot. Peroxidation of cell membranes is closely related to damages caused by some environmental stresses [22]. Enhanced MDA content under different stress conditions demonstrates that drought stress could result in lipid peroxidation induction by reactive oxygen species [23].

Considering this, the relationship between low MDA content and plant resistance to drought stress is proved in many plants such as tomato [24], cowpea [25], corn [26] chickpea [27] and canola [21]. In our study but despite of being a coordination between drought stress and MDA content, in moderate drought stress (40 percent of the soil available water content) MDA content was fallen again, so that wasn't statistically different from well-watered treatment. This reduction in MDA content after a period of rising might be attributed to the effect of enzymatic antioxidant system on reducing adverse effects of oxidative stress. Matching mean comparison of MDA content and antioxidant enzymes activity revealed that MDA content was changed in conformity with antioxidant enzymes activity. In other words, when the activity of enzymatic antioxidant system was higher (functioned more successfully), MDA content and as a result cellular damage was also lower. Therefore, MDA

content reduction in moderate drought stress level had taken place while the activity of antioxidant enzymes had reached its highest value compared with other stress levels. Accordingly second rising of MDA content in the highest level of drought stress (moisture retention at 80% of available water content) could be justified by the concurrent reduced performance of enzymatic antioxidant system. The connection between enzymatic antioxidant system and cellular damage that is usually evidenced by an enhancement in lipid peroxidation of cell membranes and MDA content has been previously reported by various authors [28-31]. A correlation between enzymatic antioxidant system and MDA content was reported in sensitive and resistant cultivars of wheat. POD, SOD, CAT and APX were all negatively correlated with MDA and H_2O_2 content [32]. Such negative correlation between enzymatic antioxidant activity and MDA content was found in our experiment (Fig. 1a-c). However APX was the only enzyme that correlated significantly with MDA content, these results can be a confirmation of the importance of the plant's antioxidant system to reduce the damage caused by the accumulation of MDA [29].

3.2 Antioxidant Enzymes

3.2.1 SOD and CAT activity

Analysis of variance showed that the activity of antioxidant enzymes was significantly affected by drought stress, however two marigold types were not different in this regard (Table 1). But mean comparison of plant types revealed that CAT activity in ornamental type was higher than medicinal one (Table 2).

The activity of two enzymes SOD and CAT was increased along with the enhancement of drought stress from control level (moisture retention at 80% of available water content). Despite this higher activity in mild water stress was not significantly different from control; afterwards it was sharply increased So that in moderate stress level, the activity of SOD and CAT was 28.5 and 30.5 percent higher than control, respectively (Table 2).

As drought stress increased to its highest level of moisture retention at 20% of available water content, the activity of both SOD and CAT was dramatically reduced to a lower level compared with well-watered control (24 and 29 percent reduction relative to control, respectively).

SOD controls the first threshold of the water-water cycle of antioxidant system [33,34]. It plays a key role in quenching active oxygen [35], working as catalyzing the dismutation of O_2- into H_2O_2 which are eliminated by CAT, POD and other antioxidant enzymes. So the observed positive correlations among SOD and CAT in our study (Fig. 1d), suggested that the increase of SOD activity was accompanied by increases of CAT activities as a result of high demand of quenching H_2O_2.

Enhanced SOD and CAT activities as a response of increasing drought stress, has been previously reported by the other authors. Salekjalali et al. [36] reported that in barley, CAT, POX and SOD activity increased as drought stress became more severe; however, the activity of APX did not significantly change in water stressed levels compared with well-watered conditions. They suggested that antioxidant protection in barley under drought stress could be attributed mainly to SOD and POX activity. When subjected to drought stress, plants respond through alteration in physiological and biochemical processes. Additionally, enzymatic antioxidant systems play an important role in scavenging harmful oxygen species. But irreversible damage occurs when the enzymatic antioxidant system under water stress is overwhelmed [31]. Plants can use the level of steady-state cellular ROS to monitor their intracellular level of stress. However, this steady-state level must be tightly regulated in order to prevent an oxidative burst by over accumulation of ROS, which would ultimately result in extensive cell damage and death [37]. In our study, reduced activity of SOD and CAT in higher levels of drought stress happened while in lower stress levels their activity was increased with increasing drought intensity. Reduced antioxidant activity of antioxidant system (CAT and SOD) in the highest level of drought which was accompanied by decreased relative water content and increased cell permeability as a result of increased lipid peroxidation of membranes (Table 2), all indicate possible damages to cell membrane [30]. The relationship between reduced activity of antioxidant enzymes SOD, CAT and POD and MDA accumulation has been previously proved by the other researchers [28,29,31]. On the one hand, a decrease in the activity of these antioxidant enzymes results in accumulation of high levels of H_2O_2 and increase in lipid peroxidation, thereby enhancing MDA

content and possibly inducing damage to membranes, whereas on the other hand, an increase in MDA level may be a feedback mechanism responding to restrain the activities of antioxidant enzymes, thus inducing more possible damage to membranes than before. This suggests that in plant tissues the antioxidant enzymes activity increases as a defensive response to water stress, but that this self-regulation level for the physiological mechanism has less and less effect with increasing drought stress [30]. In relation to the plant type on the activity of antioxidant enzymes, CAT activity in all studied canola cultivars with the exception of Licord and Zarfam as tolerant cultivars was reduced in response to drought stress. They suggested that reduced CAT activity was possibly due to the inhibition of enzyme synthesis or a change in the assembly of enzyme subunits under drought stress conditions. The higher activity of CAT in ornamental marigold tested in this experiment could be attributed to its higher tolerance to drought stress. However, when you consider the similarity of the two marigold types in other studied traits, the higher CAT activity observed may not be a strong enough indicator of the ornamental marigold's tolerance to drought stress.

3.2.2 APX activity

According to analysis of variance, drought stress levels were significantly different; however plant types didn't show any difference in terms of APX activity (Table 1). Mean comparison of drought stress levels generally showed that reduced activity of APX was accompanied by increasing drought stress. This reduction in the highest level of drought stress was up to twofold compared with non-stressed control (Table 2). These results were obtained while the most previous studies in different plants reported a decrease in APX activity in drought stress conditions. For instance, Salekjalali et al. [36] in their study on antioxidant enzymes in barley reported that APX activity was not changed with increasing drought stress, while SOD, CAT and POX were increase.

Induced enhancement of CAT and POX activity in moderate (CAT and POX) and severe (POX) drought stress levels as main scavenging agents of H_2O_2 (as a product of SOD activity) where suggested to be the reason for constant APX activity in stressful conditions. Oxidative stress increased the activity of glutathione-ascorbate cycle enzymes in rice seedlings [38]. All enzymes of this cycle including APX, dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and GR were increased by drought stress induction. However this enhancement in moderate stress (-0.5 mega Pascal) was lower than severe stress (-2 mega Pascal). Lower H_2O_2 content in moderate drought stress level is attributed to its effective scavenging by glutathione-ascorbate cycle enzymes and some non-enzymatic compounds. Considering that APX has a much higher affinity for H_2O_2 than CAT, and that CAT is primarily inactivated and then degraded in the presence of light [39], increased CAT activity with increasing drought stress intensity, along with reduced activity of glutathione-ascorbate cycle enzymes in our study, suggests an unfavorable condition for glutathione-ascorbate cycle enzymes to cope with H_2O_2 molecules. Because when there's a favorable condition for the activity of this cycle, CAT portion in scavenging these oxidative compounds is much lower than APX. In other words, depends on having ascorbate (Asc) and an active Asc regenerating system that continuously provides sufficient Asc, APX has a much higher affinity for H_2O_2 than either CAT or GPX [40]. Therefore, it is possible that insufficient Asc for APX activity caused that CAT undertake APX rule as the first line of defense against deleterious effects of H_2O_2 molecules. Sharma and Dubey [38] also demonstrated that with increasing drought stress to -2 mega Pascal, not only CAT, but the activity of glutathione-ascorbate cycle enzymes also decreased. A reduction in APX activity in higher levels of stress could be a result of increased ROS production and the reaction of these compounds with enzymes that would finally result in the oxidation and inactivation of antioxidant enzymes [41]. Higher production of superoxide anion ($O^{-}{}^{2}$), increased lipid peroxidation, reduction of reduction of soluble proteins, thiols and non-enzymatic antioxidant compounds such as ascorbate and glutathione in rice seedlings during severe stress compared with moderate stress were reported [38]. These results suggest that increased drought stress induces a severe oxidative stress in rice seedlings and it seems that the antioxidant defense system is unable to cope with the damage caused by this oxidative stress. In agreement with these results, we also concluded that reduced activity of APX to its lowest value in higher drought stress levels accompanied by reduced activity of SOD and CAT and RWC and also an enhancement in lipid peroxidation and membrane permeability (Table 2), all confirming inefficiency of antioxidant system in higher levels of oxidative stress.

Table 1. Analysis of variance on SOD, CAT and APX activity, MDA, proline and carotenoids content, RWC and flower yield of *Calendula officinalis* L

S.O.V	df	Mean Squares							
		Antioxidant enzymes			MDA	Non-enzymatic antioxidants		RWC	Flower yield
		SOD	CAT	APX		Proline	Carotenoids		
Replication	2	7.32 ^{ns}	2.06 ^{ns}	0.32 ^{ns}	0.73 ^{ns}	4.55 ^{ns}	1.61 *	4.96 ^{ns}	0.025 *
Drought stress (D)	3	12.30 *	4.11 *	2.43 **	156.26 **	1388.46 **	1.44 *	196.5 *	0.107 **
Plant type (P)	1	12.42 ^{ns}	6.13 *	0.83 ^{ns}	10.12 ^{ns}	1.10 ^{ns}	1.17 ^{ns}	52.42 ^{ns}	0.043 *
D * P	3	6.47 ^{ns}	2.06 ^{ns}	0.55 ^{ns}	22.99 ^{ns}	4.77 ^{ns}	0.27 ^{ns}	25.13 ^{ns}	0.007 ^{ns}
Error	14	2.97	0.91	0.27	20.72	4.30	0.38	35.56	0.005

The last fully expanded leaf at 50% flowering stage was used to measure superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), malondialdehyde (MDA), proline and carotenoids. Another fresh leaf soaked in distilled water for 24 hours was oven-dried to measure relative water content (RWC) at the same stage. Flower yield consisted of the whole air-dried flowers collected during the reproductive stage., ** and ^{ns} indicate significant difference with p <.05, p <.01 and not significant, respectively*

Table 2. Mean comparisons of the effect of water stress and plant type on SOD, CAT and APX activity, MDA, proline and carotenoids content, RWC and flower yield of *Calendula officinalis* L

Treatment	Antioxidant enzymes			MDA	Non-enzymatic antioxidants		RWC	Flower yield
	SOD	CAT	APX		Proline	Carotenoids		
	u.mg^{-1} protein	ΔA240.mg^{-1} protein	ΔA290.mg^{-1} protein	mmol.gr^{-1} fresh weight	mg.gr^{-1} fresh weight	mg.gr^{-1} fresh weight	%	g.plant^{-1}
Drought stress								
Well-watered	6.68 ^{ab}	3.41 ^{ab}	2.73 ^{a}	17.97 ^{a}	8.50 ^{b}	4.50 ^{a}	80.95 ^{a}	0.52 ^{a}
Mild water stress	6.82 ^{ab}	3.43 ^{ab}	1.61 ^{bc}	28.68 ^{b}	10.16 ^{b}	3.96 ^{ab}	79.98 ^{a}	0.41 ^{b}
Moderate water stress	8.58 ^{a}	4.45 ^{a}	2.23 ^{ab}	23.57 ^{ab}	35.12 ^{a}	3.85 ^{ab}	77.14 ^{a}	0.32 ^{c}
Severe water stress	5.08 ^{b}	2.42 ^{b}	1.31 ^{c}	28.67 ^{b}	36.17 ^{a}	3.30 ^{b}	68.38 ^{b}	0.21 ^{d}
Plant type								
Medicinal type	6.07 ^{a}	2.92 ^{b}	1.78 ^{a}	25.37 ^{a}	22.27 ^{a}	3.68 ^{a}	75.14 ^{a}	0.41 ^{a}
Ornamental type	7.51 ^{a}	3.93 ^{a}	2.15 ^{a}	24.07 ^{a}	22.70 ^{a}	4.12 ^{a}	78.09 ^{a}	0.33 ^{b}
C.V	25.42	27.89	26.75	18.41	9.22	15.91	7.78	20.20

Data are mean values of four replications and indicate the effect of water stress and plant type on antioxidant enzymes, non-enzymatic antioxidants, MDA and proline content, RWC and flower yield of calendula officinalis L. Well-watered: 80% of the soil available water content; Mild water stress: 60% of the soil available water content; Moderate water stress: 40% of the soil available water content; Severe water stress: 20% of the soil available water content. SOD, superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; MDA: malondialdehyde; RWC: relative water content. In each column, means with at least one similar letter are not significantly different, according to Least Significant Difference (LSD) test

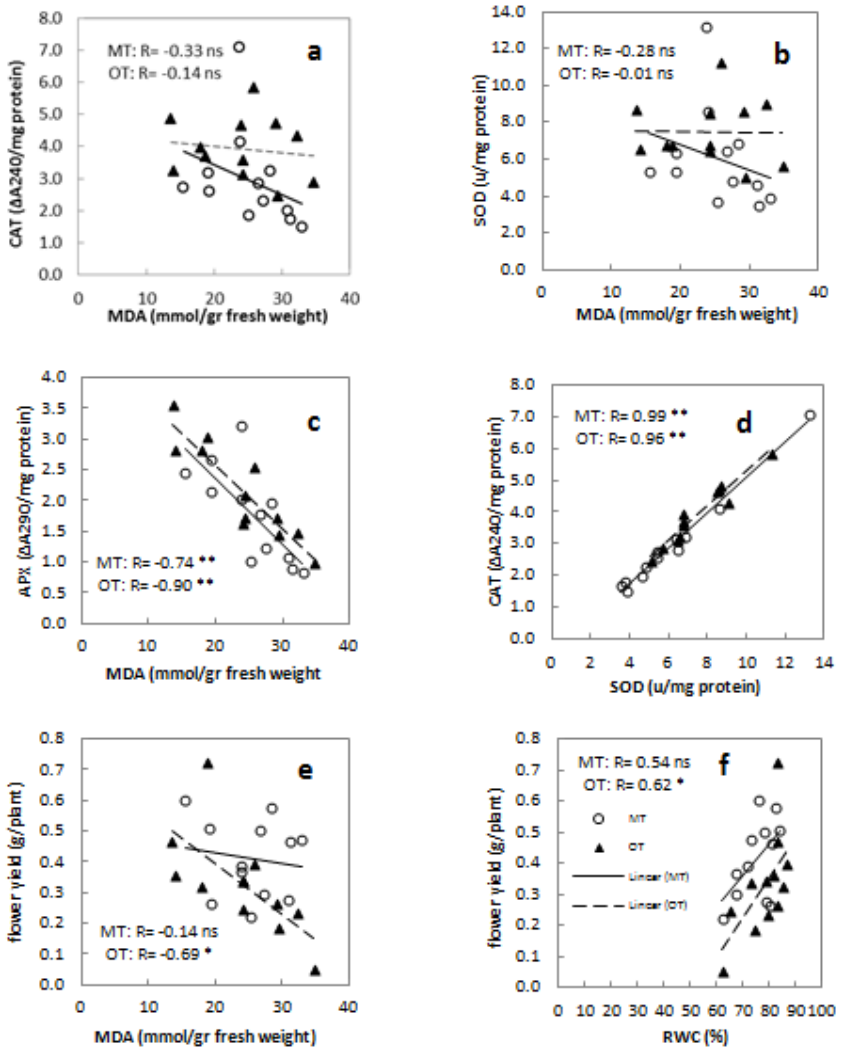

Fig. 1. Correlation between malondialdehyde (MDA) and catalase (CAT) activity (a), superoxide dismutase (SOD) activity (b), ascorbate peroxidase (APX) activity (c), flower yield (e); between SOD and CAT (d); between relative water content (RWC) and flower yield (f)

Plant type: medicinal type (MT, ○) and ornamental type (OT, ▲). The solid (—) and dashed (---) lines represent the best-fit linear regressions for each species and R is Pearson Correlation
Coefficient: * P<.05; **P<.01; ^{ns} not significant

3.3 Non-enzymatic Antioxidants

3.3.1 Proline

Proline amino acid in under drought plants was continuously increased so that its content in the highest level of drought stress (moisture retention at 20% of available water content) was up to fourfold compared with non-stressed control. However proline content in mild water stress level was not significantly different from non-stressed control, afterwards it was sharply increased with increasing drought intensity. These results correspond to those reported by Manuchehri and Salehi [42] who reported that proline content is increased with increasing salt or drought stress in bermudagrass (*Cynodon dactylon* [L.] Pers.). The highest amount of proline content was recorded in the highest intensity of drought or salt stress level. In another interesting study, Hasheminasab et al. [32] reported a negative and significant correlation

between CAT, SOD, APX and POD with MDA and H_2O_2 concentration in sensitive and tolerant cultivars of wheat. They also demonstrated that there was no significant correlation between the activity of antioxidant enzymes, cellular H_2O_2 and MDA content with proline concentration. In other words, however proline as an antioxidant agent increases as a result of oxidative stress, but there is no connection between the activity of antioxidant system and proline increasing. It might be the reason why reduced activity of antioxidant enzymes had no effect on the rate of proline enhancement in high drought stress level in our experiment (Table 2). Lum et al. [43] reported that proline content in all 8 studied upland rice cultivars continuously increased with increasing drought intensity to its highest level. However the rate of increasing in tolerant cultivars was higher compared with sensitive ones. On the one hand, these results confirm the importance of proline as a compatible solute in regulating and reducing water loss from the plant cell during water deficit [44] and that it plays an important role in osmosis balance [45], where on the other hand, it could be concluded that increased proline content is a common reaction in tolerant and sensitive cultivars under drought stress. However the rate of this increase depends on the intensity of drought stress and the degree of plant resistance to drought [43]. Accordingly, accumulation of free proline might be a general adaptation to reduce the severity of most abiotic stresses in higher plants. Although in such conditions some other amino acids accumulate too, but they are insignificant [46].

3.3.2 Carotenoids

The rate of carotenoids was decreasing with increasing drought stress, so that non-stressed control and intensive drought stress had the highest and lowest carotenoid content, respectively (Table 2).

Carotenoids are essential to protect photosynthesis against excess radiation and play an important rule as pioneers of signal transduction systems during plant development under biotic and abiotic stresses [29]. Carotenoids are also present in the plant cellular membranes. They protect the membranes from light-dependent oxidative damage. The role of Carotenoids in scavenging reactive oxygen species has been well studied [47,48]. Kabiri et al. [49] investigated the effect of drought stress (0, -0.2, -0.4 and -0.6 mega Pascal) on black seed (*Nigella sativa* L.). Reduced carotenoid content under drought stress in their study was attributed to beta-Carotene degradation and composing zeaxanthin in xanthophyll cycle. However photosynthesis pigments including carotenoids were continuously increased with increasing drought intensity in marigold (*Calendula officinalis* L.), where the highest amount of carotenoids (0.96 milligrams per gram fresh weight of petals) was obtained in the highest drought stress level [50]. Oxidative stress as a result of moisture deficit decreased carotenoids in strawberry tree (*Arbutus unedo* L.). Lutein and beta-Carotene were kept constant under moderate water stress compared with non-stressed condition, and decreased by 61 and 75% under severe stress [51]. In addition, the total amount of the xanthophyll cycle components remained unaltered under moderate stress, and increased significantly by 0.05 micromole per gram dry weight in severely stressed plants. This was mainly due to the large increases in zeaxanthin, which increased in these plants by 0.14 micromole per gram dry weight. Under severe stress, alpha-tocopherol remained at high levels, and zeaxanthin and ascorbate levels increased significantly. Together with alpha-tocopherol, the xanthophylls, and specially zeaxanthin have been ascribed a protective function in thylakoids, light- and drought-adapted plants displaying a larger pool of xanthophylls and a greater maximal conversion to zeaxanthin [52]. Beta carotene is very efficient in the scavenging of triplet chlorophyll in antenna complexes [53] and cooperates with alpha-tocopherol in the scavenging of singlet oxygen produced in the reaction center of photosystem II [54]. Thus, degradation of Beta carotene might be associated with enhanced photogenerated singlet oxygen in thylakoids [51]. Accordingly, the reduction in the total amount of carotenoids as an effective non-enzymatic antioxidant against oxidative stress in our experiment is a sign of increased degradation of these compounds in higher levels of drought-induced oxidative stress. However, no distinction has been done between different types of carotenoids in our experiment. In higher levels of drought stress, photooxidative reactions inside the chloroplast, have possibly caused damage to chloroplast membranes and resulted in degrading pigments placed on these membranes. Damage to the cell membranes indicates that xanthophylls have not done their duty in protecting cell membranes and since these membranes are the place on which carotenoids place, decomposition of carotenoids

as a result of membrane degradation is inevitable.

3.4 Flower Yield

With increasing drought, the flower yield decreased with a certain rate, so the highest (0.52 grams per plant) and lowest (0.21 grams per plant) flower yield belonged to non-stressed and intensive drought stress, respectively. Mild and moderate drought stress levels with 0.41 and 0.32 grams per plant were placed among these two levels (Table 2). Confirming these results, Rahmani et al. [55] reported that the lowest level of drought (watering after 40 millimeter evaporation from class A evaporation pan) yielded the highest dry flower (2406 kilograms per hectare) in marigold. Plant height, height of the main stem and the number of lateral branches were the highest in this level of drought, as well. Water deficit in plants may lead to physiological disorders, such as a reduction in photosynthesis and transpiration [56]. In the case of aromatic crops, significant changes in the yield and essential oils under drought stress is expectable [57]. Reduced growth and yield of pot marigold was attributed to its exposure to damaging levels of water deficit in the highest level of drought stress [58], which could result in a reduction in turgor pressure and consequently reduced cell growth and development, especially in case of stems and leaves.

Thus, the inhibition of flower growth characters under water deficit treatments would almost certainly be due to exposure to injurious levels of drought, causing a decrease of turgor which would result in a decrease of growth and development of cells [59]. Reduced water uptake decreases tissue water content and turgor pressure. Drought stress also trims down the photoassimilation and metabolites required for cell division. As a consequence, impaired mitosis, cell elongation and expansion result in reduced growth [60]. The inhibition of plant growth characters and flower yield under water deficit treatments may be due to exposure to injurious levels of drought [58] causing a decrease of turgor which would result in a decrease of growth and development of cells [61]. Cell growth is the most important process and is affected by water stress. Plant size is indicated by a decrease in height or smaller size of leaves when there is a decrease in the growth of cells. When leaf size is smaller, the capacity to trap light decreases too and the capacity of total photosynthesis decreases, i.e. photosynthesis is restricted in water shortage conditions, with a subsequent reduction in plant growth and performance [62].

So we can conclude that flower loss as the usable part of pot marigold is a result of reduction in flower yield-related traits, which is due to reduced plant growth under limited water conditions. Water deficit by reducing cell division and cell elongation and reduction of ion transport from the soil to the plant roots exacerbate growth retardation. Reduced water content indicates that cell division and cell elongation is possibly disturbed and membranes are also damaged. Positive correlation between RWC and flower yield (Fig. 1f) and negative correlation between MDA content and flower yield (Fig. 1e), clearly proves the mentioned conclusion. Analyze of variance results indicated that two studied types of pot marigold were significantly different in terms of flower yield (Table 1). Flower yield in medicinal type (0.41 gram per plant) was approximately 24% higher compared with ornamental type (0.33 gram per plant) (Table 2). Such difference was also reported in different cultivars of flax (*Linum usitatissimim* L.) which was mainly due to differences in genetic structure, growth habit and specific growth condition of these cultivars [63].

4. CONCLUSION

The results of this study clearly indicated that drought stress can considerably affect pot marigold plants. Water deficit primarily disturbed the equilibrium between producing and scavenging of ROS and resulted in damaging membrane structures of cells and enhancing MDA content and consequently simulating antioxidant system, so that SOD, CAT and APX activity and proline content was concurrently enhanced. However, carotenoids content as another important antioxidant decreased. Reduced carotenoid content with increasing drought stress intensity is possibly due to the damage to chloroplast membrane structures, on which these compounds are placed. In lower levels of drought stress, the activity of antioxidant system reduced adverse effects of water deficit, which was evidenced as reduced MDA content after a period of rising. In higher levels of drought stress however this antioxidant system failed and damages to the cells including peroxidation of cell membranes (MDA content) increased. Nevertheless, increasing rate of proline content was continued to the highest level of drought. Considering proline as an important compatible

osmolyte, increased content of this amino acid in response to drought stress is a general response to stress intensity in plants. Reflected impact of drought stress in physiological level, was as reduced flower yield of pot marigold. Flower yield decreased with increasing drought stress and reached its lower amount in severe drought stress. Negative correlation between flower yield and MDA content and RWC of cells clearly reveals that limited amount of plant dry matter due to damages to cell membranes and reduced cell turgidity and consequently reduced cell growth and cell expansion, would finally result in reduced potential of flower production in pot marigold. Despite higher potential of medicinal one, the two studied types of pot marigold were similar in other studied physiological traits. Considering these results, stimulating antioxidant system of pot marigold using different methods such as hormone implementation might improve drought resistance of this plant under water deficit conditions and improve its productivity, which indicates the necessity of such studies to be done. So it seems that if the functionality of antioxidant system of pot marigold is somehow preserved when facing higher levels of drought stress, its tolerance and production ability also increases. So higher antioxidant activity of pot marigold could be considered as a reliable index of its higher tolerance to drought in breeding programs to achieve cultivars with higher productivity under water deficit conditions.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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