The effects of glycine betaine and L-arginine on biochemical properties of pot marigold (*calendula officinalis* L.) under water stress

Fatemeh Sadat Feiz[1], Leila Hakimi[2]*, Amir Mousavi[3] and Marzieh Ghanbari Jahromi[1]

1. Department of Horticulture and Agronomy Science, Islamic Azad University, Science and Research branch, Tehran, Iran
2. Department of Horticulture, Faculty of Agriculture, Islamic Azad University, Saveh Branch, Saveh, Iran
3. Plant Biotechnology Group, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

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Abstract

Pot marigold (*Calendula officinalis* L.) is widely used due to its various biological activities to treat diseases as an analgesic, anti-diabetic, anti-ulcer and anti-inflammatory agent. To ameliorate the adverse effects of water stress on this medicinal plant, foliar application of glycine betaine (GB) and L-arginine (LA) was used. For this purpose, water stress was applied at three levels (100%, 70% , and 40% field capacity (FC)), and foliar application was used at five levels (control, 50 mM GB, 100 mM GB, 1.5 mM LA, and 3 mM LA) as factorial based on completely randomized design. We measured phenol and flavonoid content, DPPH radical scavenging activity, superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL) activity, and essential oil (EO) content and yield. Total phenol content in the interaction of 40% FC and GB 100 mM was higher than the other treatments. The highest and lowest total flavonoid content and DPPH radical scavenging activity were observed in the interaction of 40% FC and 100% FC, respectively. These variables at GB 100 mM were greater than the others. The highest SOD and PAL activity was found in the interaction of 40% FC and LA 3 mM/GB 100 mM. The content and yield of EO in the interaction of 70% FC and 100 mM GB were greater than other treatments. In sum, 70% of water stress did not significantly change the biochemical properties of *C. officinalis*, but 40% FC dramatically influenced the quality of the plant. GB 100 mM could stimulate the plant to activate its antioxidant systems under water stress and obtain the highest EO.

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Introduction

Pot marigold (*Calendula officinalis* L.), belonging to the Asteraceae (Compositae) family, is an annual plant with bright or yellow-orange daisy-like flowers which is applied for medicinal or culinary purposes (Muley et al., 2009; Fan et al., 2016). *C. officinalis* can be widely used as an antiseptic, anti-inflammatory, and cicatrizing as well as a light antibacterial and antiviral agent (Khalid and Teixeira da Silva, 2010; Metwally et al., 2013; Sak et al., 2017). A large number of

Calendula species have a characteristic scent or taste caused by mono- and sesquiterpenes within the essential oil (EO), which in many cases are the reason for their application in folk medicine (Ukiya et al., 2006). In recent years, many attempts have been made to better characterize their therapeutic characteristics and to increase the production of these useful compounds within their EOs (Khalid and Teixeira da Silva, 2012; Geneva at al., 2010). *C. officinalis* can store many carotenoids in its flowers: b-carotene, lutein, lycopene, rubixanthin, flavoxanthin, and g-carotene (Pintea et al., 2003). Metabolites can have a protective role against some types of cancer, cardiovascular diseases, and eye disorders, such as macular degeneration (Khalid and Teixeira da Silva, 2012). The primary dietary source of provitamin A is plants' carotenoids, mainly beta-carotene. The main property is their anti-oxidative activity, which protects organisms against reactive oxygenic radicals. Other components that define the antioxidant capacity consist of phenols (phenolic acids and flavonoids), water-soluble (ascorbate) and lipid-soluble (tocopherols and tocotrienols) (Geneva et al., 2010).

Environmental stresses induce wide changes in plant responses, ranging from altered gene expression and cellular metabolism to changes in growth rates and yields (Kim et al., 2015). A plethora of plant reactions exist to circumvent the potentially harmful impacts caused by an expanded range of both abiotic and biotic stresses, including light, drought, salinity, high temperatures, and pathogen infections. Among the environmental stresses, drought stress adversely influences plant growth and productivity (Fang and Xiong, 2015). knowing the biochemical and molecular responses to drought is essential for a holistic perception of plant resistance mechanisms to water-limited conditions (Kim et al., 2015). Water stress leads to a decrease in CO_2 assimilation rates due to reduced stomatal conductance. Water stress also triggers a reduction in the contents and activities of photosynthetic carbon reduction cycle enzymes, including the key enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase. The critical roles of proline and glycine-betaine, as well as the role of abscisic acid (ABA), under water stress

conditions, have been actively researched to understand the tolerance of plants to dehydration. Besides, water stress-induced generation of active oxygen species is well recognized at the cellular level and is strongly controlled at both the production and consumption levels in vivo, through increased antioxidative systems (Reddy et al., 2004).

Glycine betaine (GB) is a major organic osmolyte that accumulates in a variety of plant species in response to environmental stresses like drought, salinity, temperature, UV radiation, and heavy metals. Although its actual role in plant osmo-tolerance remains controversial, this compound thought to have positive effects on enzyme and membrane integrity along with adaptive roles in osmotic adjustment in plants grown under stress conditions. Many studies have reported a positive relationship between the accumulation of GB and plant stress tolerance (Ashraf and Foolad, 2007; Chen et al., 2011; Kurepin et al., 2015). Because of the high nitrogen to carbon ratio among the 21 proteinogenic amino acids, arginine is a main storage and transport form for organic nitrogen in plants in addition to its role as an amino acid for protein synthesis, a precursor for polyamines and nitric oxide (NO), and an essential metabolite for many cellular and developmental processes. L-Arginine (LA) is often a major nitrogen storage form also in underground storage organs and roots of plants (Nordin and Näsholm, 1997; Bausenwein et al., 2001; Rennenberg et al., 2010). Hence, arginine metabolism plays a key role in nitrogen distribution and recycling in plants (Slocum, 2005).

There is no information on the exogenous applications of GB and LA for changing the physiological and biochemical properties of *C. officinalis* L., so the present study was carried out to investigate the effect of exogenous applications of GB and LA for increasing water stress resistance of *C. officinalis* L.

Material and Methods

Site description and plant preparation

A pot experiment was carried out under greenhouse condition as a photoperiod of 16/8 (lightness/darkness) and mean temperature as 29/15° C (day/night) in the Agronomy Department

Table I
The physical and chemical properties of soil used in the study

Soil property	pH	EC (ds/m)	Clay (%)	Silt (%)	Sand (%)
Value	7.1	3.4	27	48	25

of the University of Tehran, Karaj, Iran. The seeds of *C. officinalis* were purchased from Pakan Bazr, Isfahan, Iran. After growing the seeds in coco peat + perlite (50% + 50%), the four-leaf plants were transferred to experimental pots of 17 cm diameter and 13 cm height. The soil used for experimental pots consisted of clay (27%), silt (48%) and sand (25%) with pH = 7.1 and EC = 3.4 ds/m (Table 1). A complete fertilizer containing the three main plant nutrients: nitrogen (N), phosphorus (P), and potassium (K), in the forms of potash, phosphoric acid, and nitrogen was applied twice during the experiment as 100 cc for each plant.

Treatments

The experiment was conducted as factorial based on a completely randomized design. Water stress was used at three levels (100% FC, 70 % FC, and 40 % FC). For this purpose, FC was determined by the pressure plate apparatus, and based on the FC as the control treatment (100% FC), the 70% and 40% FC were applied. The pots were weighed every day during the experiment and the deficient water was added to the corresponded pots (Xie et al., 2018). Foliar application of glycine betaine (GB) and L-arginine (LA) was applied at five levels (control, 50 mM GB, 100 mM GB, 1.5 mM LA, and 3 mM LA). Two weeks after transferring the seedlings to pots, the foliar nutrition treatments were applied in intervals of 10 days for two months. The GB and LA were applied in the morning for all plants by a hand-pump sprayer. The biochemical measurements were done in 80-day-old plants.

Traits

Extraction

The flowers of *C. officinalis* were properly dried and grind to create the powder. The powder was then extracted using methanol (1:10) in reflux condensation at 50 °C for 3 h. The obtained extracts were filtered and concentrated in a rotary evaporator. After the complete dryness of extracts, the yield value was calculated and stored for total phenol and flavonoid content and DPPH radical scavenging activity. In addition, for measuring SOD and PAL activities, leaf samples (0.5 g) were ground in liquid nitrogen before they were homogenized in extraction buffer (50 mM phosphate buffer, pH 7.0, including 1% (w/v) polyvinyl polypyrrolidone) at 4° C. The homogenate was then centrifuged at 15000 g and 4° C for 30 min. The supernatant was submitted to enzyme assays.

Total phenol content

The total phenol content in the flower extracts was determined using Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) with a little modification. Gallic acid was applied as a positive control. Samples (200 µL) were introduced into test cuvettes, and then 1.0 mL Folin-Ciocalteu reagent and 0.8 mL sodium carbonate (7.5%) were added. The absorbance of all samples was determined at 765 nm using the UV spectrophotometer after incubating at 30° C for 1.5 h. Results were expressed as milligrams of gallic acid equivalent (GAE) per gram dry extract.

Total flavonoid content determination

Flavonoid content was determined using the aluminum chloride colorimetric method (Zhishen et al., 1999). Briefly, 1 mL of flower extract was mixed with 4 mL of distilled water. Three hundred µL of sodium nitrite was added. After 5 min, 300 µL aluminum chlorides was added and allowed to stay for 6 min. Subsequently, 2 mL of sodium hydroxide was added and the mixture was shaken to mix appropriately. The absorbance was recorded at 510 nm using a UV spectrophotometer.

DPPH radical scavenging activity

Antioxidant activity was measured based on the method described by Hatano et al. (1988). Briefly, the reaction mixture contained 0.1 mL of DPPH radical solution (5 mM) and different concentrations of tested compounds (from 0.0026 mM to 83 mM). The total reaction mixture volume was 3 mL. The absorption of DPPH radical at 515 nm was determined after 10 min against a blank solution that contained methanol. % DPPH radical scavenging activity was calculated as (Ac-As)/Ac × 100 where As is the absorbance of the sample and Ac is the absorbance of the control (DPPH radical solution in methanol without sample).

Superoxide dismutase (SOD) activity

The SOD activity was analyzed in a 3-ml reaction mixture containing 50 mmol sodium phosphate buffer (pH 7.0), 10 mmol methionine, 1.17 mmol riboflavin, 56 mmol NBT, and 100 µl enzyme extract. The solution absorbance was tested by measuring its capacity of inhibiting the photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the enzyme activity that reduced the photoreduction of nitroblue tetrazolium to blue formazan by 50% (Chen and Pan, 1996). The SOD activity was expressed as enzyme units per gram fresh weight (U/g f w).

Phenylalanine ammonia-lyase (PAL) activity

PAL activity was assayed by monitoring the production of t-cinnamic acid at 290 nm (Hahlbrock and Ragg 1975). The reaction mixture contained 50 mmol Tris-HCl buffer (pH 8.8), 20 mmol L-phenyl alanine and enzyme extract. Incubation was at 30° C, and the reaction was stopped by addition of 0.5 ml 10% trichloroacetic acid. Absorbance at A nm was measured after 30 min. One unit of enzyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.01 per min. PAL activity was expressed as enzyme units per gram fresh weight (U/g f w).
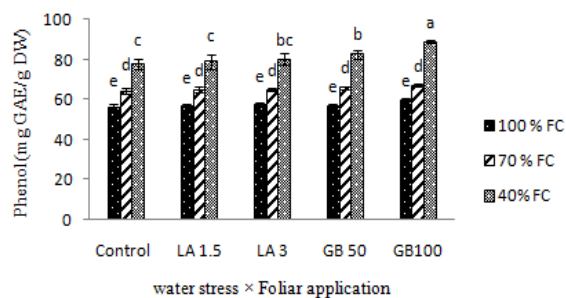
Essential oil content

Fig. I. The interaction of water stress and foliar application of GB and LA on total phenol

The essential oil content of *C. officinalis* was quantified based on the method described by European Pharmacopoeia for oil production (European Pharmacopoeia, 1983). One hundred grams of dried flowers were subjected to hydro-distillation for 3 hours using a clevenger-type apparatus. Essential oil yield was calculated as below:

EO yield= EO content × Dry weight of flowers per plant / 100

Statistical Analysis

All data were analyzed by SAS Software (version 9.2), and Duncan multiple range test was used to compare means. All data were analyzed at 5% level.

Results

Analysis of variance

Analysis of variance showed water stress and foliar application by GB and LA significantly influenced all traits (p≤0.05) (Table 2). However, the interaction of water stress and the foliar application was significant for phenol, SOD, PAL, essential oil content and yield (p≤0.05) (Table 2).

Total Phenol, Flavonoid content, and DPPH radical scavenging activity

Phenol content increased in the interaction of water stress and foliar application (Fig. I). Total phenol in 40% FC and 100 mM GB (89 mg GAE/g DW) was greater than that in the other treatments (Fig. I). The main effect of water stress and the foliar application were significant on flavonoid and DPPH radical scavenging activity

Table 2
Analysis of variance for the studied traits of *Calendula officinalis* L. under water stress and foliar application with GB and LA

S.O.V	MS								
	df	Phenol	Flavonoid	DPPH	SOD	PAL	Essential oil content	Shoot Dry weight	Essential oil yield
Water stress	2	2285**	375**	309**	2.3**	352**	0.02**	128**	0.001**
Foliar application	4	45.3**	6.7**	9.5**	0.7**	7.38**	0.008**	8.7**	0.0003**
Water stress × Foliar application	8	10.8*	1.3ns	0.82ns	0.12**	3.49**	0.0007**	0.99**	0.00002**
Error	30	4.37	1.02	1.04	4.19	1.57	0.000009	0.18	0.000002
CV.	-	3	7.77	5.9	4.06	10.3	4.7	3.4	5.6

(Table 2). The highest and lowest total flavonoid were observed in 40% FC (18.1 mg Quercetin / g DW) and control (8.1 mg Quercetin / g DW), respectively. In addition, flavonoid content was higher in all treatments of foliar application with GB and LA compared with control (Fig. II). Maximum and minimum DPPH radical scavenging activities were found in 40% FC (22.2%) and control (13.4%). DPPH radical scavenging activity in LA 3 mM as 19% was greater than that in other treatments (Fig. III). A significant and positive relationship was found between phenol, flavonoid, and DPPH radical scavenging activity (Table 3).

SOD and PAL activity

SOD and PAL activity increased in the interaction of water stress and foliar application (Fig. I). SOD activities in the interaction of 40% FC and 100 mM and also 40% FC and 3 mM LA as 5.7 U mg protein-1 min-1 were higher than those in the other treatments (Fig. IV). This trend also was observed for PAL activity (Fig. V). In addition, we observed a significant positive relationship between SOD and PAL activities (Table 3).

Essential oil content and yield

Essential oil (EO) content and yield increased by moderate water stress and foliar application. According to the dry weight of the plant (Fig. VII), we found the EO yield. The highest EO content (0.31%) and EO yield (0.046 g plant-1) were recorded in the interaction of 70% FC and 100 mM GB (Figs. VI and VIII). In addition, we observed a significant positive relationship between EO content and EO yield, whereas there was a significantly negative relationship between
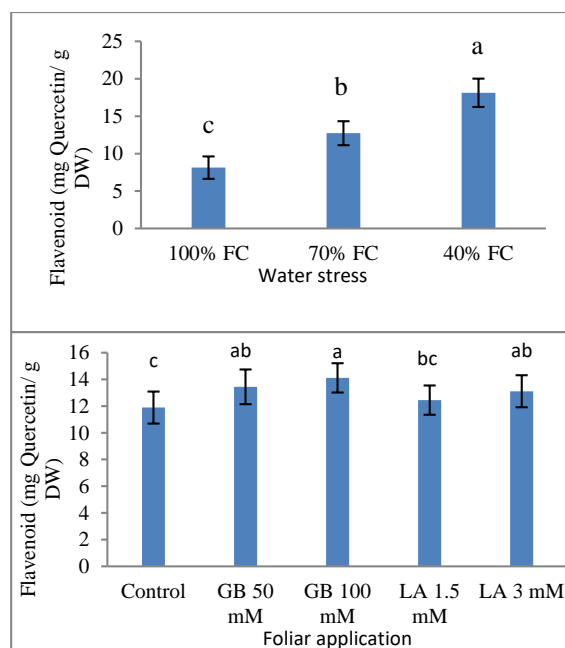

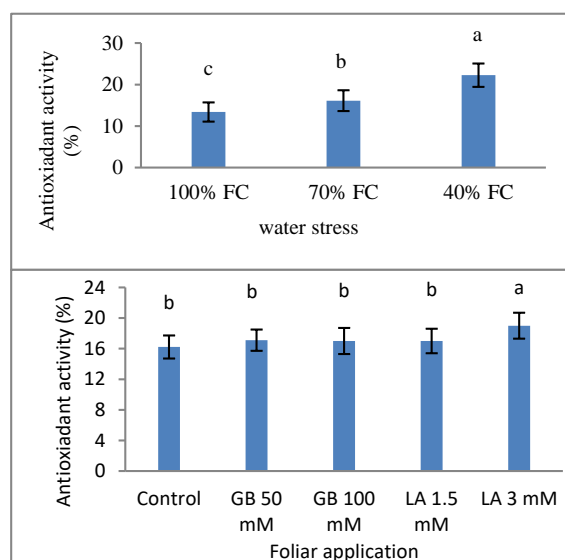Fig. II. The effect of water stress and foliar application of GB and LA on total flavonoid

Fig. III. The effect of water stress and foliar application of GB and LA on antioxidant activity

EO and EO yield with respect to SOD and PAL activity as well as DPPH radical scavenging activity (Table 3).

Discussion

Water stress, GB, and LA increased phenol, flavonoid content, and DPPH radical scavenging activity. Phenols constitute the largest group of plant secondary metabolites, varying in size from a simple structure with an aromatic ring to complex ones (Aldred, 2008; Bujor et al., 2018). In the present study, water stress and foliar application of BG increased the phenolic content. The highest phenolic content was observed in the interaction of 40% FC and 100 mM GB, which was 37% higher than that in the control (Fig. I). Phenolic compounds have a significant role in antioxidant activity in plants (Zainol et al., 2003; Krol et al., 2014; Ganesan et al., 2018). Flavonoids are a large family of polyphenolic plant compounds (Schwinn and Davies, 2018). Flavonoid content increased by water stress so that the highest value was found at 40% FC as 18.1 mg quercetin / g DW (Fig. II). We found 56% increase in flavonoid content at 40% FC as compared to 100% FC. Fifty and 100 mM GB as well as 3 mM LA increased the flavonoid content. The antioxidant activity rate is highly attributed to the structure of phenolic compounds and particularly on the number and orientation of hydroxyl groups (–OH) (Krol et al., 2014). Gharibi et al. (2016) found a positive correlation between total phenol content and antioxidant activity for different species of genus Achillea. DPPH radical scavenging was increased by increasing water stress. The highest and lowest DPPH radical scavenging was found in 40% FC and 100% FC, respectively (Fig. III). Ali et al. (2010) recommended that DPPH radical scavenging is highly due to the presence of large amounts of phenolic compounds. Besides, Weidner et al. (2000) and Ali et al. (2010) showed that phenolic compounds are stronger DPPH scavengers than flavonoids. The reduction in radical scavenging might also occur during drought stress because of damage in the cell membrane that leads to a decrease in the scavenging ability in plants and fails to bring about water-depletion (Lin et al., 2006). Water stress leads to rising reactive oxygen
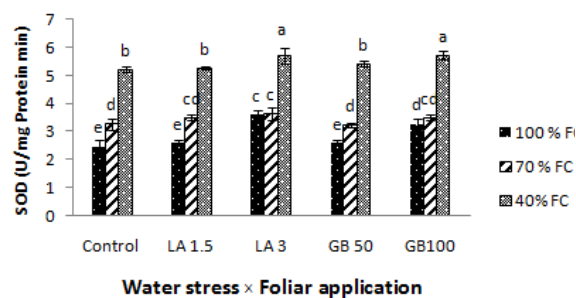

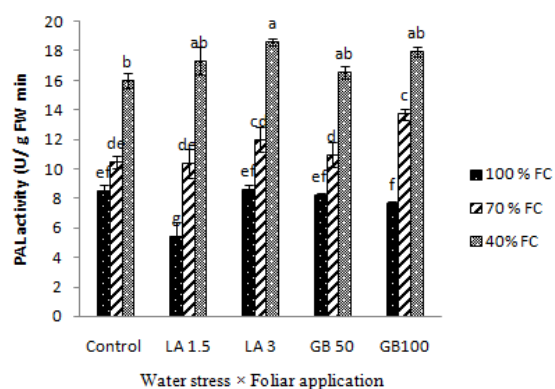Fig. IV. The interaction of water stress and foliar application of GB and LA on SOD activity

Fig. V. The interaction of water stress and foliar application of GB and LA on PAL activity

species and, therefore, greater water stress results in increasing of reactive oxygen species and, therefore, higher amounts of antioxidants are required to compensate stress condition and enhance the tolerance (Bettaieb et al., 2011). Antioxidant activity has the main role in maintaining the balance between the production and scavenging of free radicals. Moreover, the increase in phenol content under drought stress is highly correlated with the production and distribution of different antioxidants in the plant and the duration and intensity of stress (Fischer et al., 2013).

Phenol and flavonoid contents in 100 mM GB were higher than other treatments. We found a 16% increase in flavonoid contents in 100 mM GB with respect to the control. Among a large number of quaternary ammonium compounds in plants, GB occurs most abundantly in response to water stress (Mansour, 2000; Mohanty et al., 2002; Yang et al., 2003). GB is abundant mainly in chloroplast where it plays a vital role in adjusting and protecting the thylakoid membrane, thereby maintaining photosynthetic efficiency (Ashraf and Foolad, 2007). In many plants, the natural

Table 3
The Pearson correlation among variables used in the study

	Phenol	Flavonoid	DPPH	SOD	PAL	EO	Dry weight	EO yield
Phenol	1							
Flavonoid	0.93**	1						
DPPH	0.95**	0.92**	1					
SOD	0.94**	0.90**	0.92**	1				
PAL	0.90**	-89**	0.89**	0.90**	1			
EO	-0.27	-172	-215	-38**	-23	1		
Dry weight	-80**	-477**	-78**	-83**	-80**	61**	1	
EO yield	-55**	-0.78**	-496**	-0.63**	-53**	92**	86**	1

accumulation of GB is lower than sufficient to alleviate the adverse impacts of dehydration induced by different environmental stresses (Subbarao et al., 2001). Foliar application of GB to low-accumulating or non-accumulating plants may help decrease the adverse effects of environmental stresses (Yang and Lu, 2005). Externally applied GB can rapidly penetrate through leaves and be transported to other organs, where it would contribute to improved stress tolerance (Ashraf and Foolad, 2007). Because naturally produced GB does not normally break down in plants (Bray, 2000), it can easily be collected as a relatively inexpensive byproduct from high-producing plants (Rhodes and Hanson, 1993). This may make extraction and exogenous application of GB an economically feasible approach to counteract the adverse effects of environmental stresses on plant productivity.

SOD and PAL activities were enhanced by water stress, GB and LA. These enzymes activities in the interaction of 40% FC and LA 3 mM or GB 100 mM were higher than other treatments (Figs. IV and V). SOD variation depends on the severity and duration of the treatments and also the species and age of the plant (Pan et al., 2006; Ansari et al., 2018). In our study water stress increased SOD and PAL activity. Differences in protective enzyme activities are known for a number of species. Under drought stress, enhanced SOD activity was found in Salvia miltiorrhiza Bunge (Liu et al. 2011), and Amaranthus graecizans (Cunhua et al. 2011). In addition, increased PAL activity was observed for Salvia officinalis (Bettaieb et al. 2011) and Oryza sativa L. under water stress (Shehab et al., 2010). Water stress can increase reactive oxygen species (ROS) and, therefore, the antioxidant system of
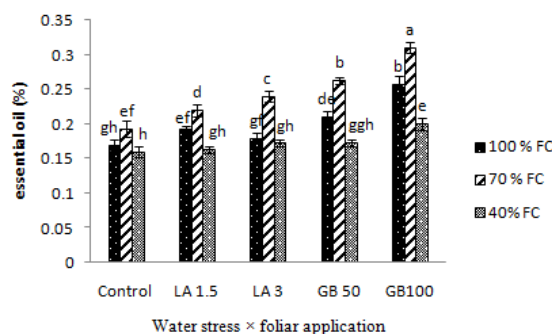

Fig. VI. The interaction of water stress and foliar application of GB and LA on essential oil content
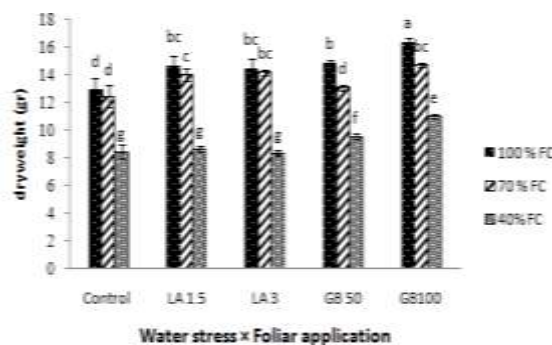

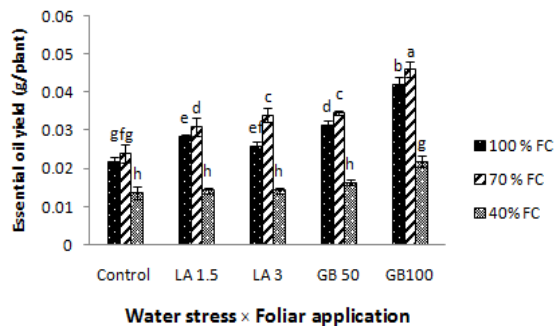Fig. VII. The interaction of water stress and foliar application of GB and LA on flower dry weight

Fig. VIII. The interaction of water stress and foliar application of GB and LA on essential oil yield

the plant will be changed (Liu et al. 2011). In our study, both SOD and PAL activity increased by enhancing water stress severity. LA as an amino acid has a significant role in alleviating the adverse effects of drought stress. As the plant imposed by stress, the amino acids like proline and LA increase (Roy and Wu, 2001). Improved growth due to foliar-applied GB under drought stress was attributed to the maintenance of requisite tissue water status (owing to the osmotic adjustment), improved photosynthesis, starch metabolism, and increased antioxidative enzyme activities (Ma et al. 2007; Rasheed et al., 2018).

The content and yield of essential oil (EO) improved by mild water stress and GB. The content and yield of EO in the interaction of 70% FC and 100 mM GB were greater than other treatments (Figs. VI and VIII). A 46% increase in EO was recorded at 70% FC and 100 mM GB compared to the control. Reduction in content and yield of EO in different plants has been reported under the influence of water stress (Razmjoo et al., 2008; Farahani et al., 2011). It was reported that the percentage of EO in mild stresses increased, but it can be decreased by severe stress. Under harsh stress, the plant produces a large amount of its photosynthetic material to produce osmotic adjusting compounds such as proline, glycine betaine and sugary compounds such as sucrose and fructose to provide the necessary conditions for their survival (Khorasaninejad et al., 2011; Mbarki et al., 2018). These compounds are costly for plants and they should be reduced by plants (Sangwan et al., 2001). Caser et al. (2016) showed that drought stress did not change the amount of essential oil of Helichrysumpetiolare, but the quality of essential oil was changed. Depending on the type and severity of the stress as well as the genetic type, we can find different strategies of plants for optimizing the amount of essential oil under stress and fertilization (Caser et al., 2016). In this research, mild stress (70% FC), was the most suitable level for obtaining the highest essential oils for *C. officinalis* L. GB due to its role in improving the plant. The negative relationship between antioxidant activity and EO content and yield might be due to the fact the plants use their energy for increasing the antioxidant activity or EO content.

Conclusion

The present study was carried out to assess the GB and LA application for ameliorating the adverse effects of severe water stress for *C. officinalis* L. Our results suggested that the antioxidant system of this plant is not significantly changed by reduction of water stress from 100% FC to 70% FC. However, to decrease from 70% FC to 40% FC, we can find a significant change in biochemical properties of *C. officinalis* L. 40% FC induced adverse effects on *C. officinalis* L., which can be ameliorated by GB 100 mM. For EO, mild stress and GB 100 mM resulted in the optimum content.

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