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Influence of ascorbic acid and 24-epibrassinolide on physiological characteristics of pot marigold under water-stress condition*

Khatereh Hemmati, Ali Ebadi, Saeed Khomari and Mohammad Sedghi

Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Mohaghegh Ardabili, Ardabil, Iran

ABSTRACT

Water deficit is considered as a major limiting environmental factor for plant growth and yield. To ameliorate the adverse effects of water restriction, an experiment was conducted in the research field of Mohaghegh Ardabili University in two successive years (2014 and 2015). Foliar spraying of different concentration of epibrassinolide (EBL) (0, 10^{-8}, and 10^{-7} M) and ascorbic acid (AsA) (0 and 10 mM) was carried out and water-stress trials included 50 and 100 mm evaporation from class A pan. Water stress significantly enhanced essential oil content, but reduced capitula yield and relative water content (RWC) of leaves. Water-stress damage ameliorated by foliar application of 10 mM AsA with 10^{-7} M EBL and the essential oil yield and antioxidant enzymes activity improved significantly. Enhancing of malondialdehyde (MDA) content and electrolyte leakage indicates that water-deficit stress caused oxidative damage to the membrane by enhancing hydrogen peroxide (H_2O_2) level. Combined-application of regulators significantly declined the amounts of H_2O_2, MDA, and electrolyte leakage under water stress. Antioxidant enzymes activity and also proline and protein content were enhanced by drought stress as well as regulators. Also, the application of EBL and AsA induced tolerance to water deficit and reduced the reactive oxygen species by increasing antioxidant enzymes activity and osmotic adjustment.

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Antioxidant defense system; ascorbic acid; pot marigold; brassinosteroid

Introduction

Pot Marigold (*Calendula officinalis* L., Asteraceae) is an annual plant from the Mediterranean and West Asia. Its aqueous extracts have anticancer properties (Kavatchev et al. 1997). The main constituents of the flowers include flavonoids, menthol, isomenthol, caryophyllene, terpenoids, esters, glycosides, and coumarins (Edward and Gilman 1999).

Plants are frequently subjected to water-deficit stress in their lifecycle. Drought stress leads to photosynthesis inhibition, cell-membrane damage, senescence, and cell death (Turkan 2011). Under water-deficit stress, reactive oxygen species (ROS) are produced due to an imbalance between light interception and its use. Additionally, ROS, causing damage to a plant cell, also act as a signal and activate some defense mechanisms against stress in living organisms (Arora et al. 2002). The production of different types of ROS-like superoxide (O_2^-), hydroxyl radical (•OH), and H_2O_2, is one of the biochemical markers that happens under environmental stress (Gill and Tuteja 2010).

Under favorable growth conditions, ROS production in a plant cell is very low but H_2O_2 and O_2^- increase under water-deficit stress (Polle 2001). ROS have detrimental effects on usual metabolism, including damages to proteins, lipids, and nucleic acids (Khan et al. 2012). Studies showed that ROS levels and defense mechanisms against them are under cell control. There are low molecular weight antioxidants such as glutathione reductase, ascorbic acid, carotenoids, and tocopherols, and antioxidant enzymes like ascorbate peroxidase, superoxide dismutase (SOD), and catalase in plant cells (Sharma and Dietz 2009) for ROS scavenging. Among antioxidants enzymes only SOD can scavenge the O_2^-, catalase and peroxidases and other antioxidants catalyze the decomposition of hydrogen peroxide to water and oxygen (Hamanaka and Chandel 2009).

Brassinosteroids (BRs) are a group of phytohormones, which are important for growth, reproduction, and plant development (Hayat et al. 2010). They are also essential in the alleviation of environmental-stress harmful effects. There is a relationship between BRs' concentration and oxidative stress in plants (Bajguz and Hayat 2009). Some reports indicate that exogenous application of BRs alters the activity of antioxidant enzymes like catalase (CAT), SOD, Ascorbate peroxidase (APX), and glutathione peroxidase (GPX), under environmental stresses (Ozdemir et al. 2004). Li et al. (1998) suggested that BRs not only enhanced the SOD, CAT, and peroxidase (POX) activity but also improved the content of ascorbic acid and carotenoids. Ascorbic acid, besides its role in cell division and metabolism, is one of the most important vitamins produced in plants and is a strong antioxidant for H_2O_2 scavenging. Ascorbic acid is found in high concentrations in chloroplast, cytosol, vacuole and the apoplastic space of leaves (Anjum et al. 2010) and may protect the antioxidant enzymes and cause enhancing their concentration. SOD scavenges the superoxide to H_2O_2 (Hamanaka and Chandel 2009). ROS accumulation under stress conditions leads to membrane damage through lipid peroxidation (Guo et al. 2005). There are various mechanisms for ROS scavenging and inhibition of lipid peroxidation including ascorbic acid and tocopherol (Anjum et al. 2010). Jin et al. (2006) demonstrated that the utilization of ascorbic acid decreased malondialdehyde (MDA) concentration and increased the SOD activity under water-deficit conditions.

CONTACT Khatereh Hemmati khhemati@yahoo.com
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There is no literature about pot marigold essential oil and ROS scavenging system in response to plant growth regulators under stress condition, so the objective of this investigation was the enhancing of the antioxidant scavenging ability and essential oil content of pot marigold by foliar application of 24-epibrassinolide and ascorbic acid under water-deficit stress.

Materials and methods

Experimental design and plant growth conditions

A farm study was conducted through the two years of 2014 and 2015 at the University of Mohaghegh Ardabili, Ardabil, Iran. The weather condition in two growing seasons has been shown in Figure 1. Only 0.79 and 0.85 mm of the 255 and 298 mm of total 2014 and 2015 precipitation occurred during the pot marigold growing period, respectively. The average temperature of the growing periods in 2014 and 2015 was 15.69°C and 15.73°C, respectively (Figure 1).

Results of the soil analysis test are presented in Table 1. The experimental field was fertilized by adding ammonium nitrate (33% N) at the rate of 125 kg ha^{-1} based on the soil test.

On 9 May 2014, and 2015, seeds were planted at 2–3 cm depth and 15 cm intra and 40 cm inter-row spacing. The plot size was 15 m^2 (3 m × 5 m). Weeds controlled by hand weeding. Irrigation regimes adjusted by irrigating at 50 and 100 mm evaporation from class A pan (as non-stressed and stressed condition respectively), which were considered as main plots and 24-epibrassinolide (0, 10^{-8}, and 10^{-7} M) and ascorbic acid (0 and 10 mM) were placed in subplots with three replicates. Water treatments were imposed at flowering stage (two months after planting date). Also, plant regulators were sprayed on foliar on the 10th day before water-stress imposing). EBL (Sigma, USA) solution was readied by ethanol. To increase regulators uptake, Tween-20 (0.05%) was used as a surfactant. Thirty days after the regulators spraying, samples were taken from the uppermost fully expanded leaves of pot marigold.

Determining the capitula yield and essential oil content

The blooms were harvested weekly at the full flowering stage and then air dried at room temperature for seven days. For determining the essential oil content, samples of 100 g of the specimens from each treatment were mixed with 500 ml distilled water and then were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil percentage was measured after dehydrating of water by anhydrous sodium sulfate (Verma et al. 2010).

The composition of essential oils

To determine the quantitative values of essential oils in pot marigold cell, a gas chromatography (Hewlett–Packard 5890) was used. The device was equipped with a flame ionization detector (FID/GC) and a capillary column composed from 5% diphenyl and 95% dimethylpolysiloxane, 30 m × 0.25 mm, 0.25-µm film thickness. The injector and detector temperatures were set to 270°C and 300°C, respectively. The helium as vector gas was balanced at 1.0 ml min^{-1} and spectra was achieved at the 20–550 m/z at two scans s^{-1}. 1 µl essential oil sample in acetone (1:100) was injected into the device (split ratio 1:10) and the quantitative analysis was done. The qualitative analysis was carried out by Agilent 6890N gas chromatography coupled to Agilent 5973N mass selective detector. The initial GC oven temperature was 60°C for 1 min and kept at 10°C min^{-1} to 180°C where it was held for 1 min, and then kept to 280°C at 20°C min^{-1} and held there for 15 min. The essential oil components were identified by authentic standards and computer-matching with NIST 05 and Wiley 275 or with mass spectra from literature.

Determining protein content

0.5 g leaf was crushed and then 3 ml of extraction buffer including 5 ml Tris-HCl 1 M, 200 µl Na2EDTA 1 M, and 0.04% of 2-mercaptoethanol was added, and the solution was centrifuged at 4000 rpm for 20 min. Then, 5000 µl of Bradford solution was mixed to 290 µl of extraction buffer and 10 µl of protein extract and vortexed, then absorbance was recorded at 595 nm (Bradford 1976).

Catalase, peroxidase, and polyphenol oxidase enzymes assays

Enzymes activity was determined based on Sudhakar et al. (2001) and Kar and Mishra (1976) proposed methods. 0.2 g

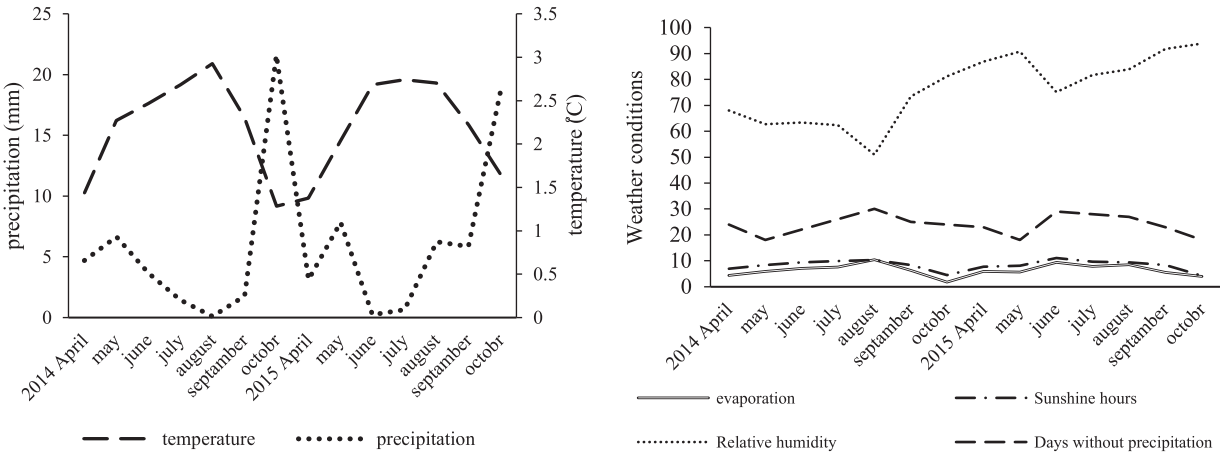

Figure 1. Monthly temperature (°C), precipitation (mm), evaporation (mm), relative humidity (%), sunshine hours and days without precipitation during the two growing seasons.

Table 1. Chemical properties of the soil.

Bulk density (g/cm^3)	pH	EC (ds/m)	Clay (%)	Silt (%)	Sand (%)	OC (%)	N (%)	P (mg/kg)	K (mg/kg)
1.23	7.5	0.72	39	30	31	0.85	0.08	2.2	95.94

of leaf samples were crushed with 1 ml of 0.05 M Tris-HCl (pH = 7.5). The homogenates were then centrifuged at 13,000 rpm for 20 min. The activity of enzymes was measured by the supernatant. For determining the catalase activity, 60 µl of the extract was added with 2.5 ml Tris-HCl buffer (50 mM) and 0.3 ml H_2O_2 (5M) and absorbance was recorded at 240 nm. 50 µl of the extract was added to the 2.5 ml buffer (Tris 100 mM, H_2O_2 5 mM, and Pyrogallol 10 mM) and absorbance was recorded at 425 nm by spectrophotometry for determining peroxidase activity. For polyphenol oxidase activity, 1.5 ml of Tris buffer at 0.2 M (pH = 7.6) and 0.3 ml Pyrogallol 0.02M were mixed and then 0.1 ml of enzyme extraction was mixed and vortexed. Then it was placed in a bain-marie for 5 min at 25° C. Absorbance was recorded at 420 nm.

Superoxide dismutase assays

0.5 g of the sample was homogenized in 10 ml of cold phosphate buffer (0.15 mol; pH = 7.5) containing EDTA (0.5 mM), and superoxide dismutase enzyme activity was measured according to Dhinsa et al. (1980). The absorbance was read at 560 nm.

Electrolyte leakage and relative water content

Leaf samples were quickly rinsed with distilled water, and one gram from the sample was immersed into 20 ml distilled water at 25°C. Then the electrical conductivity (EC1) was read of the bathing solution after 24 h. The samples were autoclaved for 20 min at 120°C. The second electrical conductivity (EC2) was recorded after the sample's equilibrium to lab temperature (Lutts et al. 1996). Then electrolyte leakage (EL) was calculated from Equation (1):

$$\text{Electrolyte leakage} = EC1/EC2*100. \quad (1)$$

Relative water content (RWC) was measured according to Weatherley (1995) procedure.

Measurement of free proline

Proline was determined in the uppermost fully expanded leaves based on Bates et al. (1973). One gram leaf sample of pot marigold was crushed in 2 ml sulfosalicylic acid (3.3%) and then centrifuged at 4000 rpm at 4°C for 10 min. In other tubes, 2 ml glacial acetic acid and 2 ml ninhydrin were mixed with 2 ml of the extract and the mixture was placed for 1 h in a bain-marie. The tubes were placed in vortex after the addition of 4 ml toluene for 15–20 s. The supernatant was used for measuring proline content at 520 nm.

H_2O_2 measurement

One gram of leaf sample was ground and 5 ml tri-chloroacetic acid 1% was added. The mixture was centrifuged for 15 min at 1200 rpm. Then 50 ml potassium phosphate buffer (10 mM, pH = 7) and 1 ml of KI (1 M) was added to the 5 ml supernatant. Absorbance was recorded at 390 nm (Heath and Packer 1968).

Determining MDA content

MDA content was measured according to McCue and Shetty (2002). 200 µl of homogenate were added in 800 µl deionized water and 500 µl of tri-chloroacetic acid 20% was mixed with 1 ml of thiobarbituric acid 10 mM. The tubes were incubated for 30 min at 100° C, and absorbance was recorded at 532 nm after centrifugation at 13,000 rpm for 10 min.

Statistical analysis

The experiment was as arranged as split factorial layout based on the randomized complete block design during the two years (2014 and 2015). The main plots were allocated to water supply and the subplots were assigned to AsA and 24-EBL. The combined analysis showed that the effect of years was not significant. Hence, the results of the two years were analyzed separately. Statistical analyses were performed by SAS software and means were compared using least significant differences (LSD5%).

Results

Essential oil percentage, capitula yield, and RWC

Water deficit significantly decreased RWC, essential oil content, and capitula yield and increased the essential oil percentage in comparison to non-stress conditions in the two growing seasons (Table 2). However, AsA and EBL improved these traits under stress and non-stress conditions. The application of EBL at 10^{-7} M + AsA at10 mM considerably enhanced yield by 50% and 48% in 2014 and 50% and 52% in 2015 in water stress affected and well-watered plants, respectively, compared to untreated plants (Table 3). Using EBL at 10^{-7} M elevated the RWC by 59% and 22% in well-watered and water-stressed plants compared to untreated plants under sufficient water and water-deficit conditions, respectively (Figure 2). Essential oil percentage elevated up to 35% and essential oil yield to 99% compared to untreated plants was achieved using EBL at 10^{-7} M and AsA at 10 mM the (Figure 3).

The composition of essential oils

In this study, the essential oils were extracted from the plant flowers and the effects of water stress, AsA, and EBL on pot marigold essential oil composition were studied. The gas chromatography profile of the oils is presented in Table 4. More specifically, a total of 39 constituents were recognized by GC–MS from the pot marigold essential oil and the recorded results indicated that the variables had significant different effects on responses. The major components of pot marigold essential oils were 1,8-Cineol, Geraniol, α-Muurolene, Delta-cadinene, Alpha-cadinol, and T-Muurolene under water-stress and well-watered conditions. However, the highest

Table 2. Effect of drought stress, ascorbic acid, and brassinosteroid on proline, protein, RWC, capitula yield, essential percentage and yield.

	Proline		Protein		RWC		Capitula yield		Essential oil content		Essential oil yield	
Treatments	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Drought stress (DS)	(μg g^{-1} FW)		(mg g^{-1} FW)		(%)		(kg ha^{-1})		(%)		(kg ha^{-1})	
Stressed	0.65	0.62	89.31	64.99	56.9	55.73	22.3	21.51	4.90	5.06	110.7	110.3
Non-stressed	0.42	0.49	69.46	86.50	78.3	79.04	43.5	43.10	3.61	3.58	159.0	155.4
LSD (5%)	0.04	0.08	2.27	5.28	10.5	5.17	2.12	2.21	1.08	0.06	36.24	7.3
AsA												
0	0.50	0.53	77.55	70.29	62.5	63.70	30.23	29.54	3.88	4.03	115.3	114.3
10 mM	0.57	0.57	81.23	81.19	72.7	70.69	35.6	35.06	4.64	4.61	154.4	151.4
LSD (5%)	0.01	0.11	1.73	4.12	3.8	6.45	1.34	1.14	0.42	0.27	10.5	8.38
EBL												
0	0.51	0.53	66.9	60.18	58.8	57.68	29.21	28.60	3.88	3.91	107.2	105.6
10^{-8} M	0.57	0.55	80.8	75.97	70.7	69.73	32.89	32.34	4.26	4.27	134.3	130.1
10^{-7} M	0.53	0.58	90.4	91.08	73.3	74.18	36.73	28.60	4.63	4.77	163.0	162.8
LSD (5%)	0.02	0.14	2.12	5.04	4.70	4.01	0.05	0.02	2.12	0.32	12.92	10.26
DS	**	ns	**	**	**	**	**	**	**	**	**	**
AsA	**	ns	**	**	**	**	**	**	ns	**	**	**
EBL	**	ns	**	**	**	**	**	**	**	**	**	ns
DS*AsA	**	*	**	ns	ns	ns	**	*	ns	ns	ns	ns
DS*EBL	**	ns	**	ns	**	ns	**	*	ns	ns	ns	ns
AsA*EBL	**	*	**	*	ns	ns	ns	**	**	*	**	**
DS*AsA*EBL	*	**	*	**	ns	ns	**	ns	ns	ns	ns	ns

*Significant at $P \leq .05$.
**Significant at $P \leq .01$; ns, non-significant.
μg g^{-1} FW = μ gram $gram^{-1}$ fresh weight.
kg ha^{-1} = kilogram $hectare^{-1}$.

amount of pot marigold essential oils under water-stress and well-watered conditions was ascribed to Delta-cadinene by 14.8 and 12.5, respectively, when 10^{-8} M EBL and 10 mM AsA were used as growth-regulating factors. Additionally, results tabulated in Table 4 showed that the treatment of pot marigold flowers under water-stress condition showed that the maximum increase in essential oils was corresponding to β-Bisabolol at 10 mM AsA + 0 M EBL (57.7%) and 1,8-Cineol at 10^{-8} mM EBL + 0 M AsA (89.1%), 10^{-8} EBL mM + 10 M AsA (89.3%), 10^{-7} mM EBL + 0 M AsA (118.9%), and 10^{-7} mM EBL + 10 M AsA (148.8%). On the other hand, the treatment of pot marigold flower under well-watered condition exhibited that the maximum increase in essential oils was related to 1,8-Cineol at 10 EBL + 0 mM AsA (34.4%), 10^{-8} mM AsA + 0 M EBL (64.4%), 10^{-8} AsA mM + 10 M EBL (90%), 10^{-7} mM AsA + 0 M EBL (122.2%), and 10^{-7} mM AsA + 10 M EBL (146.6%).

Proline and protein content

Results showed that co-application of plant growth regulators induced different changes in proline content, especially proline concentration enhanced under drought stress by using AsA and/or 24-EBL. There was 12% and 21% increase in 2014, only by EBL application at 10^{-8} M on non-stressed plants compared to the untreated plants under non-stressed and drought-stressed conditions, respectively. The application of EBL and AsA considerably increased the protein content. In both growing seasons, the maximum protein content (96% and 24% in 2014 and 111% and 79% in 2015) was observed by using10 mM AsA + 10^{-7} M 24-EBL compared to the untreated plants under drought stress and non-stressed conditions, respectively (Table 3).

Oxidative damage and antioxidant defense system

Drought stress affected pot marigold plants had the highest activity of the antioxidant enzyme (CAT, SOD, Polyphenol oxidases (PPO) and POX) compared to non-stressed plants (Table 5). However, treating the water-stressed plants with EBL and AsA enhanced the CAT, SOD, PPO, and POX activity. Co-application (10 mM AsA + 10^{-7} M 24-EBL) was the most effective treatment, which enhanced CAT, PPO

Table 3. Effect of brassinosteroid and ascorbic acid application on proline, protein, and capitula yield, under drought stress and non-stress condition.

			Proline		Protein		Capitula yield	
	EBL	AsA	2014	2015	2014	2015	2014	2015
Treatments	(M)	(Mm)	(μg g^{-1} FW)		(mg g^{-1} FW)		(kg ha^{-1})	
Drought stress	0	0	0.61	0.69	44.4	41.4	17.4	16.6
		10	0.45	0.56	78.9	61.9	20.3	22.0
	10^{-8}	0	0.63	0.45	82.5	84.3	22.5	22.9
		10	0.46	0.44	52.9	50.7	23.9	20.0
	10^{-7}	0	0.61	0.62	70.9	64.2	23.6	22.6
		10	0.45	0.70	87.4	87.4	26.2	24.9
Non-stressed	0	0	0.66	0.64	84.9	61.1	34.7	33.7
		10	0.39	0.43	88.4	90.2	44.5	37.2
	10^{-8}	0	0.74	0.62	86.3	82.9	37.7	44.8
		10	0.41	0.44	85.7	87.6	47.5	44.0
	10^{-7}	0	0.68	0.71	85.2	87.5	45.6	47.6
		10	0.38	0.45	105.4	109.7	51.5	51.3
LSD (5%)			0.17	0.11	4.03	10.13	3.20	2.88

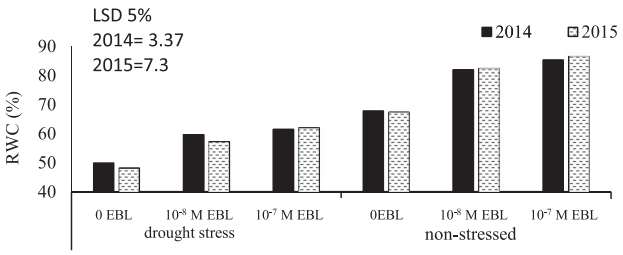

Figure 2. Effect of brassinosteroid on leaf relative water content (RWC) under drought stress and non-stressed condition at heading stage.

SOD, and POX activity under drought-stressed condition (Table 6).

Co-application (10 mM AsA + 10^{-7} M EBL) increased CAT, POX, and SOD activity of drought-stressed plants by 85%, 60%, and 34% in 2014 and 75%, 57%, and 33% in 2015, respectively, and that of by PPO by 126% and 101% in both years, respectively, compared to control. Also, co-application (10 mM AsA + 10^{-7} M EBL) under non-stress condition (Table 6) led to the high elevation of POX and PPO activity nearby 2-fold in both seasons compared to the untreated plants. Drought stress caused significantly increases the electrolyte leakage, MDA and H_2O_2 content (Table 5). Nonetheless, a different pattern of regulation was indicated when these independent variables were investigated in AsA and/or 24-EBL, under different water supply conditions. Application of EBL and AsA significantly decreased the electrolyte leakage, MDA, and H_2O_2 contents. EBL treated plants showed the minimum electrolyte leakage (5% and 7% during 2014 and 2015) under water-stress conditions. Mixed-application (10 mM AsA + 10^{-7} M EBL) in water-stressed plants considerably decreased the MDA content by 52% in both seasons and that of H_2O_2 by 52% in 2014 and 2015 compared to untreated plants under drought stress condition also non-stressed plants showed significantly decrease in MDA and H_2O_2 content nearby 5-fold decrease in both seasons compared to the control (Table 6).

Discussion

Essential oils, also known as a type of secondary metabolites, are highly complex mixtures of volatile compounds (Tabatabaie and Nazari 2007). Variations in essential oil contents of plant tissue could be attributed to the different factors such as genetic properties and environmental conditions (Gomes et al. 2013). Elsewhere, quantitative characteristics of essential oils in a plant depend on climatic effects, such as water availability and soil nutrients (Lima et al. 2003). Based on literature reviews, *C. officinalis* blooms contain high volatile components (Okoh et al. 2008). The results indicated that drought stress increased the essential oil percentage and decreased capitula and essential oil yield of pot marigold. It suggested that the amount of essential oil would increase under water stress and presence of EBL and AsA. From the viewpoint of essential oil enhancement, these data evidently suggested that the water stress could be more influential than the well-watered condition and induced tough changes in the volatile content. Simon et al. (1992) showed that the essential oil content and its components were strongly influenced by abiotic stresses. Although drought stress increased metabolites that prevent oxidative agents in plant cells, however, it caused the decrease of essential oil yield, because the collaboration between the flower yield and essential oil content is pivotal as two components of the essential oil yield (Pirzad and Shokrani 2012). The components showed higher amounts in this study were nearly constituent with the findings of (Khalid and Teixeira 2012) in pot marigold essential oils. Delta-cadinene was the compound demonstrated the maximum amount in pot marigold structure. Consistently in our result, Jianu et al. (2016) reported that the most abundant essential oil in pot marigold was Delta-cadinene.

Plants have the different antioxidant capacity to respond and adjust to water deficit. Also, researchers suggest that calendula oil has great potential to scavenging the ROS (Guinot et al. 2008; Butnariu and Coradini 2012). In many species, BRs (Ahammed et al. 2013) and ascorbate (Kalantar Ahmadi et al. 2015) regulated the defensive responses of plants to different stresses. Reports revealed that 24-epibrassinolide (Swamy and Rao 2009) and ascorbic acid (Reda et al. 2007) influenced the volatile content and quality of medicinal plants. Furthermore, 24-epibrassinolide (Radhakrishnan and Lee 2013) and ascorbic acid (Anjum et al. 2010) regulate antioxidant mechanisms under various stress conditions. Our result indicated that using co-application of AsA and 24-EBL was an effective method for mitigating the harmful effects caused by water deficit. Water stress significantly impaired the capitula yield, essential oil content and RWC in 2014 and 2015. However, ascorbic acid mitigated the negative effects of water deficit and significantly increased the essential oil percentage, capitula yield, and RWC in 2014 and 2015. Low capitula yield and essential oil content performance under water deficit could be attributed to over-generation of hydrogen peroxide, which caused injury to the membrane

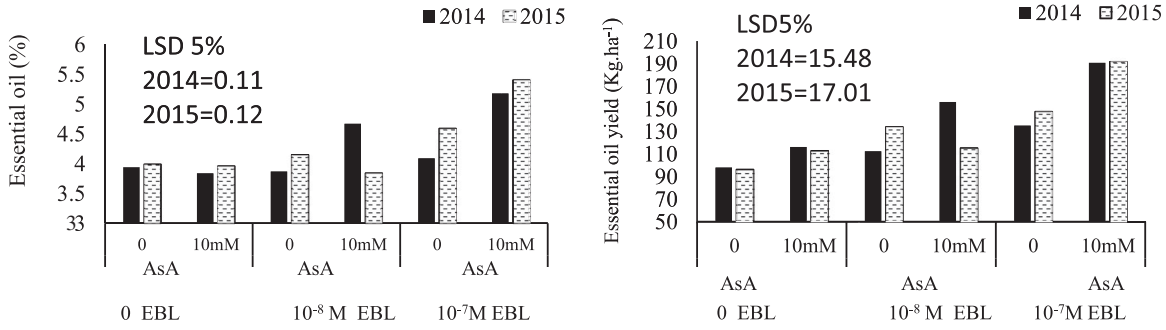

Figure 3. Effect of brassinosteroid and ascorbic acid on essential oil and yield.

Table 4. Chemical composition of pot marigold essential oils.

		Water stress						Well-watered					
	EBL (M)	0		10^{-8}		10^{-7}		0		10^{-8}		10^{-7}	
Compound	AsA (mM)	0	10	0	10	0	10	0	10	0	10	0	10
2,4-dimethyl-2,4 heptadiene		1.65	1.30	1.45	1.25	1.20	1.32	1.53	1.13	1.00	0.90	0.95	0.97
Tricyclene		1.30	0.30	1.68	0.90	1.85	1.30	1.15	0.00	1.42	0.75	1.30	0.80
α-Thujene		1.33	1.50	1.60	1.90	1.95	2.02	1.35	1.42	1.38	1.80	2.15	2.50
α-Pinene		2.05	2.05	0.90	1.90	1.35	1.35	2.30	2.43	1.65	2.30	1.70	1.72
Comphene		2.07	1.86	2.05	1.80	2.40	2.00	1.70	1.50	2.08	1.62	1.90	1.55
β-Pinene		1.57	1.67	1.50	1.40	1.07	1.11	2.20	1.80	2.00	1.70	1.40	1.50
Sabinene		2.25	2.40	1.85	1.80	1.45	1.50	2.08	1.63	2.10	1.60	1.77	1.40
γ-2-Caren		1.40	1.48	1.47	1.50	1.83	2.10	1.47	1.60	1.75	2.20	2.00	2.70
Myrcene		2.00	1.90	2.37	2.05	1.50	1.60	2.20	2.00	1.75	1.13	1.50	0.87
α-Phelandrene		1.90	2.10	2.00	2.25	2.35	1.40	1.45	1.80	1.50	1.80	1.45	1.45
Limonene		1.90	1.70	2.13	2.35	2.10	2.37	1.30	1.35	1.50	1.87	1.63	2.30
1,8-Cineol		3.95	4.75	7.47	7.48	8.65	9.83	4.50	6.05	7.40	8.55	10.0	11.1
β-Ocimene		1.65	2.05	2.10	2.10	2.20	2.40	1.25	1.15	1.40	1.50	2.05	1.73
γ-terpinene		1.85	1.85	1.72	1.00	1.20	0.80	2.37	2.30	2.40	2.25	1.45	1.25
p-cymen		1.35	1.85	1.75	1.80	2.23	2.05	1.30	1.70	1.30	1.63	1.40	1.10
Terpinolene		1.75	1.10	1.47	1.40	1.32	1.00	2.13	2.03	1.85	1.75	1.87	1.33
Fenchone		1.90	1.95	2.05	1.93	1.20	1.75	1.72	1.62	1.53	1.60	0.95	1.47
Geraniol		7.32	9.40	6.70	9.55	8.20	9.15	4.70	5.30	5.20	6.55	5.90	8.25
Menthone		1.35	1.40	1.47	1.80	1.67	1.97	1.40	1.60	1.74	1.92	1.95	2.10
Citronellel		1.95	1.73	1.50	1.45	1.50	2.20	2.23	1.73	2.10	2.20	1.45	2.23
Comphore		1.45	1.55	1.73	1.21	1.85	1.49	1.70	2.00	2.08	1.58	2.15	1.80
Linalool		1.35	1.40	1.55	1.70	1.50	1.85	1.85	2.40	2.15	2.15	2.00	2.20
α-Burbonene		1.95	1.85	2.13	1.00	2.33	1.05	1.63	1.73	1.85	0.95	1.70	1.10
B-Elmene		1.30	1.73	1.23	1.65	1.23	1.54	1.75	2.09	1.67	1.82	1.60	1.70
α-Humullene		1.60	1.10	2.20	1.45	2.12	1.58	2.23	1.63	2.33	1.73	2.43	2.10
Gama-Gurjunene		1.30	1.42	1.40	1.23	1.55	1.23	1.60	1.57	2.15	1.20	1.70	1.53
α-Muurolene		5.10	7.80	6.30	8.15	7.10	7.55	4.50	5.75	4.20	6.30	4.25	5.25
Sigma-cadinene		2.50	4.03	2.25	2.20	1.90	2.00	1.70	2.05	1.85	1.95	1.55	1.95
Delta-cadinene		9.95	12.7	10.7	14.8	12.1	13.0	7.60	8.75	8.85	12.5	8.75	9.85
Beta-calacrorene		1.65	1.05	2.02	1.52	2.15	1.55	1.80	1.30	2.60	1.45	2.20	1.73
Nerolidol		1.15	1.35	1.50	1.50	1.35	1.75	1.33	1.37	1.55	1.93	1.35	1.95
Guaiol		1.43	2.00	1.87	1.27	2.05	1.88	0.75	1.00	1.00	1.00	1.20	0.90
Beta-Acrenol		1.63	1.35	1.60	1.75	2.15	2.30	1.10	0.95	0.95	1.40	1.72	2.32
Bulnesol		1.05	1.05	1.90	1.50	1.40	1.95	1.85	1.45	1.95	2.00	1.90	2.00
Alpha-cadinol		8.05	11.5	9.15	11.70	9.85	11.0	6.10	6.75	7.55	10.05	6.75	8.05
T-Muurolene		4.90	3.80	5.55	4.05	6.10	4.20	7.10	6.25	7.50	4.95	6.80	6.15
Spathulenol		1.50	0.00	1.25	0.55	1.50	1.15	1.75	1.60	1.55	0.90	1.70	1.60
β-Bisabolol		0.90	1.42	1.40	1.45	1.05	1.50	1.37	1.50	1.40	1.70	1.72	1.75
Pentacosan		0.90	1.10	1.25	1.00	1.10	1.25	1.00	1.25	1.30	1.10	1.85	1.50

and enhanced the MDA concentration (Table 2). ROS has harmful effects on physiological processes and directly damages DNA, proteins, and lipids, which lead to cell fade out and reduction of capitula yield (Turkan 2011). The interaction between water stress, EBL, and AsA applications improved plant development. The increase of capitula yield by co-application under water deficit is an outer symptom of internal modification in plant metabolism. Induction of

Table 5. Effect of drought, ascorbic acid and brassinosteroid on catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), polyphenol oxidase (PPO) activity; hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content.

	CAT		POX		SOD		PPO		H_2O_2		MDA		Electrolyte leakage	
Treatments	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Drought stress (DS)	OD per mg protein min								($\mu M\ g^{-1}$ FW)		($\mu M\ g^{-1}$ FW)		(%)	
Stressed	56.9	43.6	93.5	92.7	22.3	365.4	43.8	79.7	0.42	0.56	0.28	0.32	10.7	11.07
Non-watered	78.2	30.3	48.8	46.4	43.6	271.4	29.0	58.9	0.23	0.18	0.13	0.17	2.9	3.13
LSD (5%)	4.5	0.22	4.4	13	39.79	30.73	2.85	6.6	0.01	0.03	0.03	0.02	1.01	0.63
Ascorbic acid (AsA)														
0	35.2	35.6	61.7	62.3	357.4	300.6	62.4	60.2	0.42	0.46	0.26	0.30	7.56	7.12
10 mM	37.6	38.3	80.5	76.8	330.8	336.2	79.0	78.4	0.24	0.29	0.15	0.18	6.17	7.08
LSD (5%)	2.29	2.12	2.19	3.63	11.24	15.8	3.02	1.97	0.01	0.03	0.04	0.02	0.47	0.56
Brassinosteroid (EBL)														
0	30.7	32.5	62.9	61.3	343	326.5	57.1	56.5	0.34	0.48	0.24	0.33	9.98	9.98
10^{-8} M	35.0	35.6	67.6	66.5	346.9	310.8	73.9	72.2	0.35	0.35	0.19	0.23	5.47	6.27
10^{-7} M	43.5	42.7	82.9	80.8	342.3	318.0	81.2	79.2	0.30	0.29	0.17	0.18	5.15	5.05
LSD (5%)	2.81	2.5	2.68	4.4	13.77	19.32	3.70	2.2	0.02	0.03	0.05	0.02	0.58	0.68
F significance														
DS	**	**	**	**	**	**	**	**	**	**	**	**	**	**
AsA	**	*	**	ns	**	**	**	**	**	**	**	**	**	ns
EBL	*	**	**	**	ns	**	**	**	**	**	**	**	**	**
DS*AsA	**	ns	**	**	ns	ns	**	**	**	**	ns	*	**	ns
DS*EBL	ns	**	ns	**	**	**	ns	**	**	**	ns	*	**	**
AsA*EBL	ns	ns	**	*	ns	**	**	**	**	**	ns	**	**	*
DS*AsA*EBL	*	ns	*	**	**	*	**	**	**	**	**	**	**	**

*Significant at $P \leq .05$.
**Significant at $P \leq .01$; ns, non-significant.

Table 6. Effect of brassinosteroid and ascorbic acid on antioxidant enzymes activity, H_2O_2 and MDA content under water stress.

Treatments			POX		SOD		PPO		CAT		MDA		H_2O_2		Electrolyte leakage	
	EBL	AsA	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
	(M)	(Mm)	μmol H_2O_2 decomposited $min^{-1}mg^{-1}$ protein								($\mu M\ g^{-1}$ FW)		($\mu M\ g^{-1}$ FW)		(%)	
Water stressed	0	0	72.3	71.0	355.3	338.7	42.7	46.71	31.2	33.1	0.67	0.76	0.67	0.76	17.8	15.33
		10	72.9	69.7	368.7	347.0	86.3	80.72	42.0	38.7	0.66	0.75	0.66	0.75	11.2	10.95
	10^{-8}	0	108.1	111.0	378.7	351.3	89.4	89.39	57.3	54.8	0.44	0.50	0.44	0.50	8.0	7.09
		10	94.5	93.5	400.0	344.3	80.3	78.15	33.9	34.5	0.56	0.63	0.56	0.63	14.5	16.50
	10^{-7}	0	96.6	99.2	382.3	359.0	91.1	88.96	40.9	43.1	0.34	0.39	0.34	0.39	5.1	8.00
		10	116.2	111.9	475.7	452.3	96.9	94.19	57.7	58.0	0.32	0.36	0.32	0.36	8.2	8.52
Non-stressed	0	0	35.3	37.8	372.7	283.0	42.3	39.66	30.0	32.7	0.29	0.33	0.29	0.33	3.9	4.14
		10	35.7	38.3	361.0	257.0	53.6	50.72	24.7	28.1	0.16	0.18	0.16	0.18	2.4	2.88
	10^{-8}	0	46.0	45.9	308.0	226.7	59.9	54.00	26.4	26.3	0.20	0.22	0.20	0.22	2.0	2.33
		10	49.4	42.9	244.0	339.7	62.9	61.27	28.0	30.1	0.19	0.21	0.19	0.21	3.6	3.96
	10^{-7}	0	64.9	58.9	275.7	280.3	64.6	68.48	32.5	32.5	0.07	0.08	0.07	0.08	3.3	3.24
		10	61.1	54.7	207.0	241.7	78.5	79.33	32.7	32.2	0.06	0.07	0.06	0.07	2.3	2.26
LSD (5%)			5.43	9.02	29.04	36.88	7.83	5.43	5.57	2.06	0.03	0.06	0.03	0.06	1.26	1.26

tolerance by foliar applications is due to increasing CAT, SOD, and POX activity (Tables 5 and 6), reduction in H_2O_2, MDA content and electrolyte leakage (Table 5), increasing proline and protein accumulation (Table 2). Therefore, this investigation could suggest that treating plants with the above-mentioned plant regulators activate the detoxification pathways which contain the essential oil and activity of the antioxidant mechanism, because calendula essential oils have antioxidant potential (Butnariu and Coradini 2012) and can improve water deficit tolerance by enhancing antioxidant defense system.

Since pot marigold is a medicinal plant, the enhancing of its essential oil is very important for the pharmaceutical industry, and our study indicated that the application of EBL and AsA by the augmentation of the antioxidant defense system can improve the essential oil content in pot marigold. Although water stress leads to oxidative damage by generating ROS, the activity of antioxidant enzymes support in protecting the equivalence in capitula yield, essential oil content, and scavenging of ROS (Gill and Tuteja 2010). Results in Tables 5 and 6 showed that SOD, CAT, and POX activities increased under water stress, but these enhancements of enzymes activity were not enough for scavenging of ROS, because MDA and H_2O_2 concentration simultaneously increased (Table 6). Co-application of AsA and 24-EBL on water-stressed plants improved the antioxidant enzymes (Table 6). Increased activity of CAT, SOD, and POX might effectively detoxify detrimental ROS effects which were clearly suggested by a considerable reduction in MDA and H_2O_2 concentration of calendula. This phenomenon was also indicated by Radhakrishnan and Lee (2013).

Obviously, SOD is the first enzyme of the ROS scavenging system, which converses the O_2^- (superoxide radical) to O_2 and H_2O (Gill and Tuteja 2010). Also, CAT altered the commutation of H_2O_2, a strong and detrimental oxidizing factor to $H_2O + O_2$ (Mittler 2002; Gill and Tuteja 2010).

Malondialdehyde (MDA) is identified as an appropriate trait for lipid membranes peroxidation (Mittler 2002). As shown in Table 5, water deficit enhanced MDA content significantly and it had a strong relationship with H_2O_2 accumulation and electrolyte leakage in two growing seasons. Also, Table 6 indicates that co-application (EBL and AsA) caused lower MDA, H_2O_2, and electrolyte leakage level in pot marigold leaves under the same water-stress conditions. Reduced H_2O_2, MDA, and electrolyte leakage by co-application of EBL and AsA could be related to maximum stress tolerance in pot marigold plants. This study revealed that treated plants with EBL and AsA gain the higher potential to alleviate the oxidative stress that reduces their sensitivity to water deficit. This phenomenon was indicated by Radhakrishnan and Lee (2013), Huang et al. (2014). Prior reports showed that using of 10 mM AsA caused a decrease in H_2O_2 and MDA content and electrolyte leakage. Our result showed that co-application of EBL and AsA can act directly as stress-preservation compounds to improve the capability of the plant antioxidant defense system by reducing the H_2O_2, MDA content, electrolyte leakage, and induction of proline and protein synthesis in stressed plants, which could be beneficial to improve plant capitula and essential oil yield (Tables 5 and 6 and Figure 3). According to the interaction between AsA and 24-EBL, our result supports the phenomenon that application of 24-EBL cooperated beneficially with exogenous AsA, stimulating plant capitula yield and essential oil content also enhancing the activity of antioxidant enzymes and proline and protein accumulation in water-stress conditions.

Accumulation of soluble protein and osmolytes regulate plant tolerance to abiotic stresses (Ma and Turner 2006). Ascorbic acid is one of the main co-enzymes for some enzymes on photosynthetic-related proteins and reduces the protein degradation and reduction under water stress (Dennison et al. 2001). Also, osmoprotectants can directly scavenge ROS or protect antioxidant enzymes. It is suggested that proline can neutralize the single oxygen during osmotic stress and inhibits damages such as lipid peroxidation, which comes from ROS (Matysik et al. 2002; Wang et al. 2009). Lipid peroxidation decreased with the application of AsA and EBL, probably through proline increase.

Furthermore, Bray (1997) suggested that there was a significant negative relation between RWC and proline content under drought stress conditions, and leaf dehydration led to an increase in proline concentration (Lazcano-Ferrat and Lovatt 1999). Plants increase their proline content under various stresses and this solute helps in the osmotic adjustment of cells (Kavikishor et al. 2005) and even inhibits oxidative damages (Wang et al. 2009), but its increase is sometimes not sufficient (Ashraf and Foolad 2007). So, application of some compounds like AsA and EBL could help stress tolerance due to elevating in proline accumulation. Literature also showed increasing of proline after AsA (Rezaee et al. 2013) and EBL (Anuradha and Rao 2007) application. It seems that EBL application increases proline synthesis and

it protects the antioxidant enzymes and helps to their activity. Furthermore, researchers showed that proline has antioxidant activity, protecting redox balance and cellular homeostasis, activating ROS scavenging pathways, energy source of the stress-recovery process, the role of protein precursor, and as a signaling compound (Islam et al. 2009; Szabados and Savoure 2010).

We achieved the aim of increasing the essential oil content and improving pot marigold tolerance to water deficit by inducing antioxidant defense system. Based on our findings, it can be concluded that using of AsA and EBL exert valuable effects on calendula's antioxidant capacity, essential oil content and capitula yield and some osmolytes under water-deficit stress that lead to improved essential oil content and capitula yield. Finally, a considerable increase in essential oil content by EBL and AsA application may be of great relevance to productivity in water-stress conditions, as a scavenger of free radicals and for reducing oxidative damage.

Disclosure statement

No potential conflict of interest was reported by the authors.

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