Australian Journal of Crop Science AJCS

AJCS 10(11):1496-1503 (2016) ISSN:1835-2707
doi: 10.21475/ajcs.2016.10.11.PNE19

Effect of brassinosteroid (24-epibrassinolide) on morphophysiological parameters and essential oils of *Calendula officinalis* L. by EC nutrient solution

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Abstract

This study evaluated the effect of different levels of electrical conduction of nutrient solution on the morphophysiological parameters of *Calendula officinalis* L. and the interaction of electrical conduction of the nutrient solution and spray of 24-epibrassinolide with three electrical conduction (EC) levels of nutrient solution (1.5, 3, and 4.5 dS.m^{-1}) and three hormone levels of 24-epibrassinolide (0, 0.5, and 1 µM) on *C. officinalis* L. under hydroponic conditions. The experiment arranged as factorial with completely randomized designs and four repetitions. Based on the results of the test with different EC levels, the nutrient solution showed significantly affected the studied parameters and diameter of *C. officinalis* L. (P<0.01). The traits of flowering stem height, wet weight, dry weight and volume of root, number of flowers, total phenol, total flavonoid, relative water content of leaf and stability of the petal's cellular membrane were significantly affected by treatment with brassinosteroids, and interaction of EC of the nutrient solution and spray of brassinosteroids. On this basis, the maximum number of flowers was observed in the treatment with EC=1.5 dS.m^{-1} and EBR=0.5 µM. The maximum total flavonoid was observed with EC=4.5 dS.m^{-1} and EBR=1 µM and the minimum rate was observed in EC=4.5 dS.m^{-1} and hormone control treatment (with no application of brassinosteroids hormone). The increase of EC in the nutrient solution led to an increase in alpha-cadinol, delta-cadinene and sigma–cadinene. Based on the results of the experiment, the application of the optimal concentration of nutrient solution and spray on acinolide is effective in helping improve morphophysiological parameters, and increases the flavonoid compounds and ingredients of *C. officinalis* L.

Keywords: *Calendula officinalis* L., Essential oil, Medicinal plant, 24-epibrassinolide.
Abbreviations: *C. officinalis* L._ *Calendula officinalis* L.; EC_ Electrical Conduction.

Introduction

Pot marigold **(***Calendula officinalis* L.) is from the annual Astracea family and has sunflower-like flowers with orange, yellow, or bright colors that have various applications, including in pharmaceuticals and spices (Bcerentrup and Robbelen, 1987; Chromak and Smith, 1988). The main compounds of *C. officinalis* L. include terpenoids, phenol acids, flavonoids, isorhamnetin, carotenoids, glycosides, sterol, and vitamin C (Re et al., 2009; Anderson, 2001). Optimal concentration of mineral elements in plant medium is one of the most important factors in the performance and quality of horticultural crops (George and Robert, 2012). Increases in concentration of mineral elements in nutrient solutions to meet the needs of plants and increase the performance and quality of the crop leads to the challenge of increased osmotic potential (salinity) of the nutrient solution. Given this, determination of a suitable level of electrical conduction in the nutrient solution has a prominent role in determining the developmental parameters and quality of the essential oil. On this basis, Tabatabaie and Nazari (2007) studied different levels of electrical conduction (EC) of nutrient solution and showed that different levels of EC had considerable effects on the growth parameters of peppermint *(Mentha piperita)*. In *M. piperita*, the maximum wet and dry weight of the plant were observed after use of the EC=1.4 dS.m^{-1} treatment. The brassinosteroids groups of plant hormones have considerable biological effects on plants, including the increase in resistance to abiotic stresses, control of flowering, and improvement of performance (Hayat and Aqil, 2011). Considering the importance of increasing access of food elements concurrently with increasing the osmotic potential of the nutrient solution, and the different reactions of different plants to this two-fold approach, this experiment was designed and conducted to study the effect of different levels of electrical conduction of nutrient solution on developmental parameters and also control the role and interaction of brassinosteroids with EC of the nutrient solution.

Result

Plant height

Considering the results obtained from analysis of variance (ANOVA), interaction between electrical conduction of the nutrient solution and the brassinosteroids hormone on the height of the plant was significant. Comparison of the means showed that the maximum height of the plant is correlated with electrical conduction of EC=1.5 dS.m^{-1} and application of 0.5 µM of 24-epibrassinolide (Figure 1).

Wet and dry weight and volume of root

As shown in Table 1, analysis of variance (ANOVA) showed that the interaction of the nutrient solution's degree of electrical conduction and brassinosteroids hormone on wet weight, dry weight, and volume of the root was significantly different at the 0.01 level of probability. Means comparison (Table 1) showed that the maximum wet and dry weight and volume of the plant root is related to treatment of EC=3 $dS.m^{-1}$ with no hormone application; the minimum value of wet and dry weight of the plant root is related to electrical-conduction treatment of EC=4.5 $dS.m^{-1}$ and application of 1 μM of brassinosteroids hormone; and the minimum root volume is related to electrical-conduction treatment of the nutrient solution of EC=1.5 $dS.m^{-1}$ and application of 1 μM of brassinosteroids hormone.

Flower Diameter

The interaction of electrical conduction in the nutrient solution and brassinosteroids hormone in determining the diameter of the flower was significant to a probability level of 1% (*P*=0.01). According to the results obtained from comparison of the means, the minimum diameter of the flower is related to electrical conduction of EC=3 $dS.m^{-1}$ and no application of brassinosteroids hormone, and electrical conduction of the nutrient solution of EC=1.5 $dS.m^{-1}$ and no application of the brassinosteroids hormone and other treatments had no significant difference from each other (Table 1).

Number of flowers

The results of the ANOVA test showed a significant effect of electrical conduction of the nutrient solution and 24-epibrassinolide on the number of flowers was significant to a probability level of 1% (*P*=0.01) The minimum number of flowers was related to electrical conduction of EC=4.5 $dS.m^{-1}$ and no application of the brassinosteroids hormone; the maximum number, with electrical conduction at EC=1.5 $dS.m^{-1}$ and a hormone concentration of 0.5 μM (Table 1).

Stability of cell membrane

The interaction of the electrical conduction of the nutrient solution and the concentration of the brassinosteroids hormone on cell-membrane stability was significant (Table 1). Comparison of the means showed that the maximum ion leakage of the plant occurred with electrical conduction of the nutrient solution of EC=4.5 $dS.m^{-1}$ and concentration of brassinosteroids hormone of 0.5 μM; minimum ion leakage of the plant was obtained with electrical conduction of the nutrient solution of EC=1.5 $dS.m^{-1}$ and no hormone application (Figure 2).

Relative water content of leaf

Interaction of electrical conduction of the nutrient solution and the brassinosteroids hormone with relative water content was significant (Table 1). with respect to the means comparison (Table 2), the maximum relative water content of the leaf in electrical conduction was with *EC=1.5* $dS.m^{-1}$ and hormone concentration of 0.5 μM; minimum relative water content in electrical conduction of the nutrient solution was *EC=1.5* $dS.m^{-1}$ and the hormone concentration was 1 μM.

Total phenol

ANOVA revealed a significant interaction for electrical conduction of the nutrient solution and the brassinosteroids hormone and the total phenol of the plant.

The minimum number of flowers was related an electrical conduction of 4.5 $dS.m^{-1}$ with no application of the brassinosteroids hormone, and the maximum number of flowers in electrical conduction of nutrient solution was obtained with 1.5 $dS.m^{-1}$ and a concentration of hormone of 0.5 μM *(*Table 1*)*. Considering ANOVA, interaction of electrical conduction of the nutrient solution and the brassinosteroids hormone on total phenol of the plant was significant. With respect to the means comparison, the maximum relative weight of total phenol of the plant in electrical conduction of the nutrient solution was 1.5 $dS.m^{-1}$ and concentration of hormone was 0.5 μM, and the minimum relative content of total phenol in electrical conduction of the nutrient solution was 4.5 $dS.m^{-1}$ and concentration of hormone was 1 μM (Figure 3).

The ANOVA results showed that the interaction of electrical conduction of the nutrient solution and brassinosteroids hormone, and electrical conduction of the nutrient solution with total phenol of *C. officinalis* L. was significant at the 0.05 level. The maximum value of this trait in electrical conduction of the nutrient solution was 4.5 $dS.m^{-1}$ with application of 1 μM of brassinosteroids hormone; the minimum value, in electrical conduction of the nutrient solution of 1.5 $dS.m^{-1}$ with the absence of hormone application (Figure 4).

Constituents of the flower extract

Table 2 shows that the interaction of electrical conduction in the nutrient solution and 24-epibrassinolide was effective in determining the content of the phenol compounds in the *C. officinalis* L. extract. These phenol compounds included alpha-cadinene, sigma-cadinene, and alpha-cadinol; the nutrient solution of 4.5 $dS.m^{-1}$ and hormone concentration of 1 μM provided the highest electrical conduction values.

Discussion

Plant height depends on growth environment. Since growth phenomenon results from the biotic activities in conditions where plants should have enough water, height will be reduced in the case of failure to supply the required water due to reduction of turgor pressure of the growing cells and will affect the length of cells (Munns and Tester, 2008). As mentioned above, plant height depends on the amount of accessible water in the plant. In this study, plants were tallest with electrical conduction of 1.5 $dS.m^{-1}$ and application of *0.5* μM of 24-epibrassinolide (Figure 1); and the maximum amount of water in the plant was obtained with the same treatment (Table 1). The minimum plant height was obtained in electrical conduction of 3 $dS.m^{-1}$ with 24-epibrassinolide hormone; in this treatment, the minimum amount of water absorption was found in the plant. In studies by Sadat Noori et al., (2006), plant height in wheat was significantly reduced with salinity stress. Increase of electrical conduction of the nutrient solution led to increase in osmotic pressure resulting from accumulation of nutrient elements, and finally reducing water absorption and nutrient elements along with plant height. In our study, Table 1 shows that maximum wet and dry weight and volume for the roots were obtained with electrical conduction of nutrient solution of 3 $dS.m^{-1}$ and no application of the brassinosteroids hormone. This may be due

Table 1. Comparing mean effect of electrical conduction of nutrient solution, 24-epibrassinolide and their interaction with morphophysiological traits of *C .officinalis* L.

EC (dS/m)	EBR (µM)	R.F.W (gr/b)	R.D.W (gr/b)	R.V (gr/cm^3)	F.D	F.N (cm)	R.W.C (µM/gr.f.w)
1.5	0	39.23^{b}	4.82^{bc}	43.75^{b}	5.13^{bc}	3.00^{e}	66.89^{de}
	0.5	19.66^{cd}	2.21^{de}	17.50^{c}	6.55^{a}	6.5^{a}	83.23^{a}
	1	16.23^{de}	1.96^{de}	17.50^{c}	5.95^{ab}	3.25^{ed}	58.33^{e}
3	0	79.29^{a}	10.16^{a}	76.25^{a}	4.5^{c}	4.00^{c}	78.94^{abc}
	0.5	37.79^{b}	3.53^{cd}	42.50^{b}	7.00^{a}	3.75^{cd}	70.09^{bcd}
	1	14.10^{ef}	2.10^{de}	25.00^{c}	6.906^{a}	5.25^{b}	60.25^{de}
4.5	0	42.90^{b}	5.94^{b}	42.25^{b}	6.386^{a}	2.00^{a}	75.54^{abc}
	0.5	23.44^{c}	2.34^{de}	20.00^{c}	6.05^{ab}	5.25^{b}	83.01^{a}
	1	10.21^{f}	1.50^{e}	18.75^{c}	6.58^{a}	4.00^{c}	68.29^{cde}

EC - Electrical Conduction; EBR – Epi Brassinolide; RFW – Root Fresh Weight; RDW – Root Dray Weight; RV – Root Volume; FD - Flower Diameter; FN - Number of flowers; RWC - Relative water content of leaf; The treatments which have common letters in the column do not have significant difference from each other based on Duncan's Multiple Range test *at* the5% level of probability.

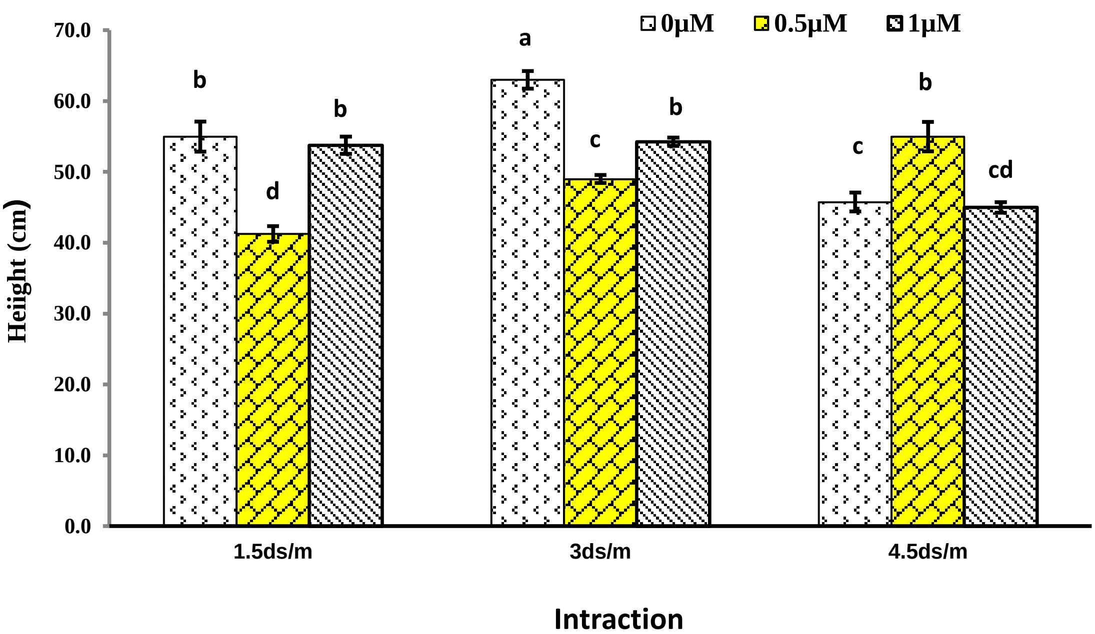

Fig 1. Effect of spray of Brassinosteroids on height of *C. officinalis* L. affected by different levels of electrical conduction of the nutrient solution

to the fact that phosphorus plays an effective role in the root growth of the plants, and phosphorus absorption increases with increasing of the nutrient solution's electrical conduction to 3 dS.m^{-1}. While plant growth also increased, more increases in the concentration of nutrient elements, particularly nitrogen with a conductivity of the nutrient solution of 4.5 dS.m^{-1}, resulted in the acidification of the root environment, reduced root growth, and (potentially) stoppage of root growth. Application of 24-epibrassinolide showed an interaction with the auxin hormone and had a synergistic effect with the cytokinin hormone. Increased concentration of the auxin hormone in plants enhances the rate of the cytokinin hormone, negatively impacting the rooting of the plant and reducing growth rate. By extension, the brassinosteroids hormone has a negative effect on rooting. Swami and Rao (2010) mentioned that an increase in different salinity levels reduces the diameter and yield of the flower *Plectranthus forskohlii* (Wild) Briq. (Syn. *C. forskohlii*). Salinity reduces flower diameter, the weight of the aerial organ, and the yield of flowering barley plants (Mercure et al., 2004). In electrical conduction of 3 dS.m^{-1}, the highest flower diameter was obtained by increasing calcium absorption by the plant and application of 0.5 µM of 24-epibrassinolide. The reason for this increase is the stimulation of growth of the brassinosteroids hormone (Swami and Rao, 2010). Potassium (K) plays an important role in crop growth and development, metabolism, and yield formation (Marschner et al., 2012). On the other hand, ammonium and potassium, the two main cations, compete with calcium absorption in plant and reduce absorption of calcium from nutrient solutions (Woodson et al., 1982). In this research, the increase in concentration of nutrient elements increases calcium absorption rate, reducing potassium absorption. Accordingly, the highest flower yield was observed with electrical conduction of 1.5 dS.m^{-1} and application of concentration of 0.5 µM of the hormone with the highest potassium absorption. In many studies, the presence of brassinosteroids hormone has been considered as a factor that assists with the reduction of ion leakage in plants under salinity stress. Korkmaz et al. (2007) reported that brassinosteroids reduced ion leakage and stomatal conductance under drought stress in *Cucumis melo* L. Results of other tests showed that the application of brassinosteroids in hypocotyls of *Cucurbitaceae* and under cold stress

Table 2. Value of ingredients based on treatments in terms of percent of total extract of *C. officinalis* L.

EC ($dS.m^{-1}$)	1.5			3			4.5		
Br (μM)	0	0.5	1	0	0.5	1	0	0.5	1
Alpha-Pinene	2.6	0.9	2.9	1.5	1.8	2.6	3.1	3.6	0.8
Camphene	1.9	2.8	2.0	0.9	2	1.5	2.2	1	0.5
Sabinene	0.8	1.4	2.6	3.4	2.6	2.9	0.9	2.4	1.3
Myrcene	2.8	1.8	2	1.6	1.7	1.5	2.6	2.2	0.5
Fransen	2.2	2.4	1.2	2.6	3.1	0.9	1.8	0.9	1.5
Humulene alpha	0.7	1.9	1.4	3.9	0.6	1.8	0.9	1.1	0.4
Gamma Gurjunene	2.5	1.3	2	1.6	1.8	2.7	3.9	1.7	0.9
γ-Muurolene	1.6	1.9	1.5	3.1	4.5	3.3	3	1.4	1.3
Sigma cadinen	5.4	6.2	7.8	7.1	7.8	8.3	7.7	8.6	9.5
Delta cadinen	17.1	19.2	21.1	19.4	21.4	22	21.5	23.3	24.2
Beta padcolen	2.8	1.9	2.6	2.9	1.4	2.2	0.8	1.8	1.2
Beta carolen	4.6	3.8	2.9	2.3	1.3	0.9	2	3.3	5.2
Nerolidol	5.2	4.4	3	2.1	1.4	1.6	3.9	2.5	6.5
Beta akronel	4.4	3.9	4	2.6	0.8	3.4	2.7	1.8	4.6
Alpha endensmol	1.6	2.9	3.3	3.6	4.1	3.3	1.9	3.3	0.7
Bolenzol	3.3	3.8	2.6	1.9	2.9	2.5	2.9	2.7	0.6
Alpha cadinol	24.2	26.2	28.8	26.5	29	28.3	28.5	32.5	36.1
Isosedranol	2.4	3.6	0.9	1.8	1.7	2.4	1.6	0.9	0.4
bisabolol	3.7	2.8	4	2.4	3.6	1.7	1.4	1	0.8
Pentacosane	2.8	3.5	2.1	4.2	3	2.1	3.3	2.5	1.3

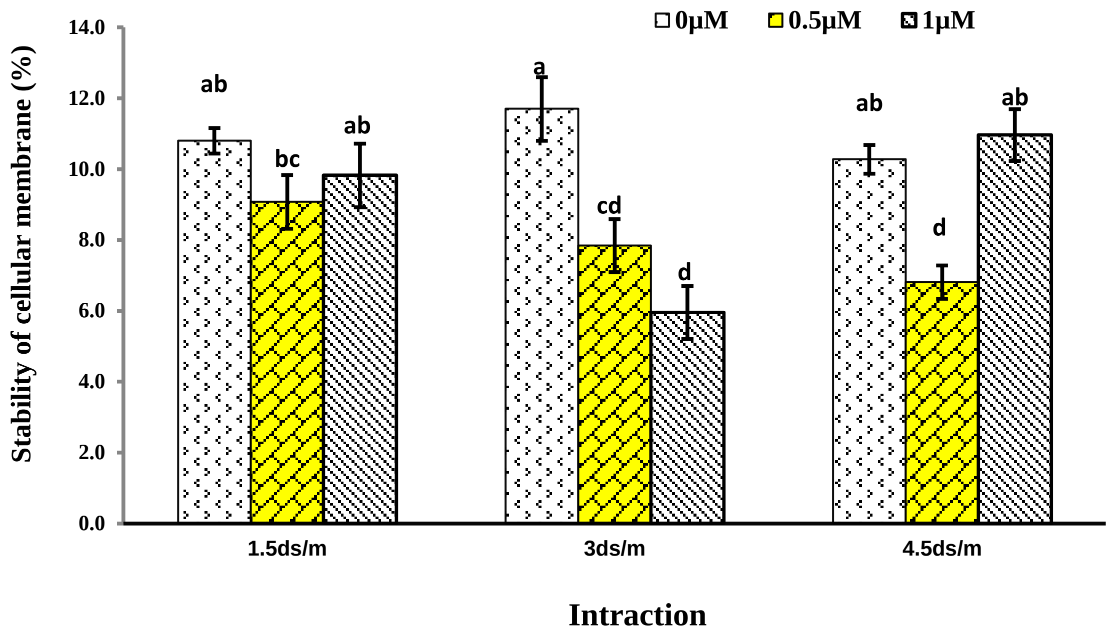

Fig 2. Interaction of Brassinosteriods hormone and electrical conduction of the nutrient solution with ion leakage of *C. officinalis* L.

conditions caused reduction of ion leakage (Kang and Saltveit, 2001). Figure 2 shows that minimum ion leakage was obtained in electrical conduction of 4.5 $dS.m^{-1}$ with hormone concentration of 0.5 μM. As indicated, accumulation of sugar in plants reduces damage resulting from ion leakage, and the highest sugar accumulation was obtained with this treatment.. Having physiologically positive turgidity can be regarded as an important adjustment strategy for preventing water shortage in leaves (Meneguzzo et al., 2000). Another mechanism plants apply for preserving turgidity and maintaining water amount in salinity stress conditions is the effectiveness of the internal structure. In this regard, analysis of the vascular region leads to resistance of diameter and the number of vesicles in water flow course. An increase in pore-less and watery parenchyma or in the thickness of cuticles and the formation of trichomes reduces evaporation and transpiration (Hanson et al., 2007). The presence of brassinosteroids hormone at concentrations of of 0.5 μM in all three electrical conduction treatments (1.5, 3 and 4.5 $dS.m^{-1}$) led to a maximum water absorption rate, while the application of a 1-μM concentration of 24-epibrassinolide showed a descending trend in water absorption rate in all three treatments. The abscisic acid plant hormone plays an important role in many growth aspects, such as regulation of pore opening at the beginning of response and adjustment to different environmental conditions (drought adjustment, low temperature, low salinity), during which the combined activity of abscisic acid

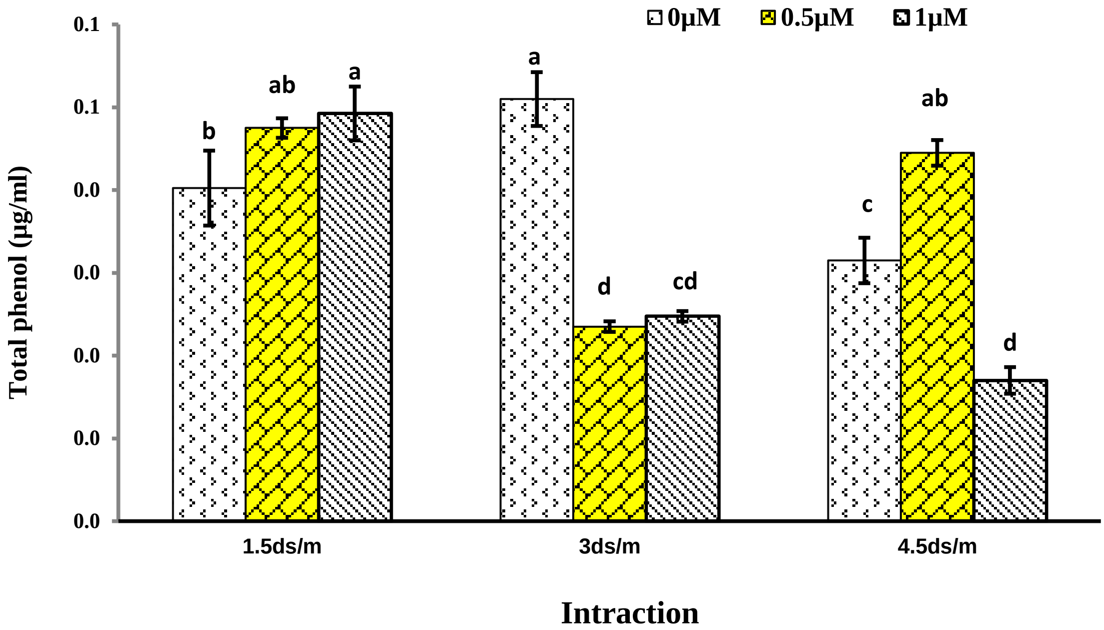

Fig 3. Effect of Brassinosteriods hormone and electrical conduction of the nutrient solution on total phenol of *C. officinalis* L**.**

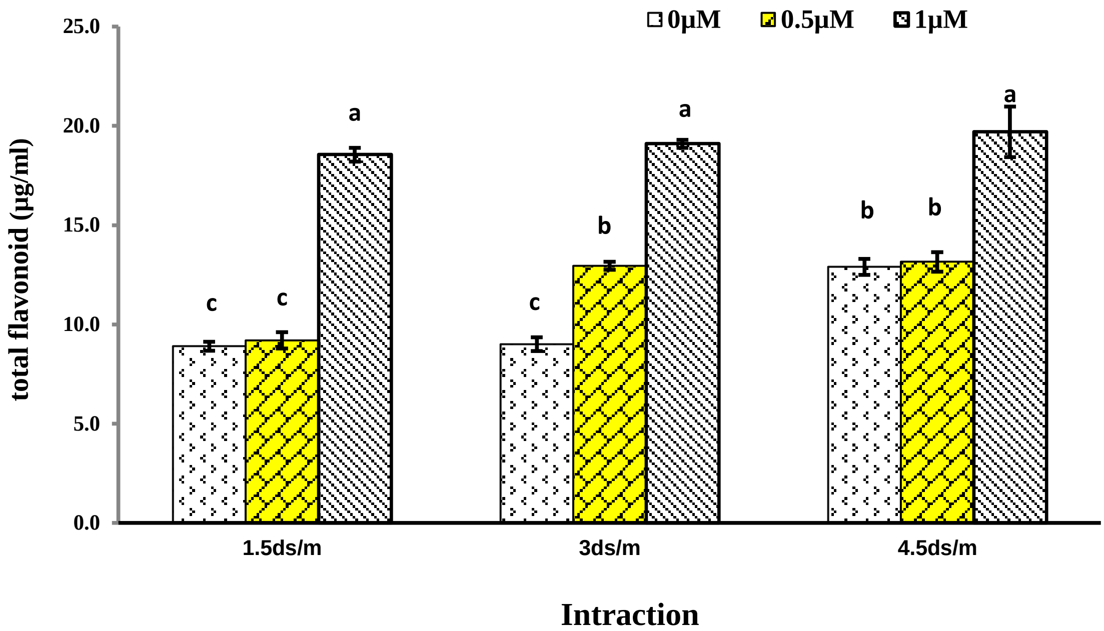

Fig 4. Effect of Brassinosteriods hormone and electrical conduction of the nutrient solution on total flavonoid of *C. officinalis* L.

and dependent and interrelated signal paths are regulated (Shinozaki et al., 2003). Due to the antioxidant properties of the brassinosteroids hormone, the plant does not experience water shortages when this hormone is applied in stress conditions, particularly in drought stress. Therefore, abscisic acid rate does not increase, and concentrations of 24-epibrassinolide increase in the plant, reducing the abscisic acid hormone rate and reducing the plant's water absorption. Phenol compounds can neutralize free radicals due to the presence of hydroxyl groups and can act as hydrogen or electron donors (Fukumoto and Mazza, 2000). Synthesis of phenol acids increases antioxidative activity and reduces free radicals in the plant. This is due to ability of the phenol compounds to neutralize hydroxyl radicals (Wettasinghe and Shahidi, 2000). The use of brassinosteroids can help increase phenol compounds in the plant due to stresses, which is due to the accumulation of carbohydrates (Mazorra et al., 2002). These researchers showed that the use of brassinosteroids increases phenol compounds in the plant. The results of the present study agree with results of Mazorra et al. (2002), considering that the phenol rate increases during times of stress; this ascending trend for the phenol rate can be justified by the effect of the brassinosteroids hormone in increasing plants' resistance to stress. Herbs are rich in secondary metabolites and serve as ingredients of many medicines. Although these ingredients have the potential of conduction of genetic processes, their production is affected by environmental factors. These factors cause changes in the growth of herbs and also in the value and quality of their ingredients, such as alkaloids, steroids, volatile oils (extracts), etc (Yanive and Palevitch, 1982). Food elements such as nitrogen naturally cause changes in yield of crop with its effect on vegetative and reproductive growth of herbs and also affect quality and quantity of their ingredients. In addition, the presence of synergistic properties of food elements leads to changes in absorption of some unnecessary elements for the plant, and these elements are mostly poisonous and hazardous to man (Grundwald and Buttle, 1996). Therefore, a fertilizer should be recommended for the herbs, considering the above cases, because a fertilizer treatment may increase the crop yield while reducing the amount of ingredients of herbs or changing the quality of the constituents of the ingredients, which may not be useful, or fertilizer treatment may improve the amount of crop and amount of ingredients of herbs while it increases the amount of heavy and poisonous elements (such as cadmium or lead) or other harmful compounds (such as nitrate), which is clearly undesirable (Grundwald and Buttle, 1996). Results of these studies also agree with those of Chalchat et al. (1991) and Gazim et al. (2008). Studies have shown that environmental factors and stressful factors affect chemical compounds and extracts from plants. For example, increase in the content of the extract in peppermint leaf has been reported to be influenced by osmotic stress (Charles and Simon, 1990). Although direct studies on the effect of electrical conduction of the nutrient solution and its effect on the flavonoids of *C. officinalis* L. were not found, different studies have been conducted that reported on the change in flavonoid compounds in different plants affected by different stresses (Mercure et al., 2004; Hashiba et al., 2006). Studies on flavonoid compounds and their role in absorption of ultraviolet beams in plants have shown that salinity pretreatments leads to increases in the rate of produced flavonoids. Fedina et al. (2006) suggested that flavonoid increase rate is an indicator of stress. On the other hand, the present study shows that salinity stress in the mentioned limit increases flavonoid rate, and the mentioned ingredients can help plants tolerate different stresses (such as salinity) due to the resultant antioxidant properties. Dastmalchi et al. (2007) showed that water-soluble compounds are isolated from the *Dracocephalum moldavica* L. containing antioxidant properties and that this antioxidant activity results from hydroxycinnamic acids and flavonoids. As noted earlier, the production of secondary metabolites (and thus the medicinal properties) of herbs can be affected by environmental factors and stressors in spite of their genetic source. Medicinal products can degrade biotically or abiotically in soils and water, a process that in general reduces their potency, even if some degradation products might be persistent and thus of concern (Mudgal et al., 2013). Therefore, environmental factors cause changes in growth of herbs and also the quantity and quality of ingredients such as alkaloids, steroids, and volatile oils (extracts) (Yanive and Palevitch, 1982). Nutrient elements (such as nitrogen) that affect the vegetative and reproductive growth of herbs also change crop yield and affect quantity and quality of their ingredients. In addition, the presence of synergistic properties of food elements leads to change in absorption and unnecessary elements in the plant; these are mostly hazardous/poisonous to man (Grünewald and Buttle, 1996). The aforementioned cases strongly argue in favor of a fertilizer for herbs, which could increase crop yield while reducing the amount of undesirable ingredients in herbs and perhaps changing the quality of constituents of herbs that may not be useful. Fertilizer treatment could also improve the amount of the crop and of herbs' useful ingredients while still increasing the amount of heavy and poisonous elements (cadmium, lead, other harmful compounds), which is clearly not desirable (Grünewald and Buttle, 1996). Results of these studies also agree with results of the study of Chalkato Chalchato et al. (1991) and Gasium, (2008).

Materials and Methods

Conditions of the experiment and studied treatments

This greenhouse experiment utilized a factorial design based on completely randomized design with two treatments and four replications. Experimental treatments included the electrical conductivity of nutrient solution of macronutrients at three levels (1.5, 3, 4.5) $dS.m^{-1}$ and foliar application of 24-epibrassonolide in three levels (0, 0.5, 1) μM on ornamental plant marigold.

Preparation and cultivation of pot marigold

Plant materials

The research was conducted in the spring and summer of 2013 in hydroponic greenhouses at the Department of Horticulture Science and Research at Azad University, Tehran Province. between latitude In 51 degrees and 6 minutes up to 51 degrees and 38 minutes of eastern longitude and 35 degree and 34 minutes up to 35 degree and 51 minutes North latitude is located and its height from sea level between 800 meter in the north up to 1200 meter in the center and 1050 meter in the south is variable. . This study was conducted on *C. officinalis* L. from the annual Astracea family as a factorial experiment based on completely randomized design with two factors of EC and EBR and four repetitions. The treatments included different levels of EC of nutrient solution (1.5, 3 and 4.5 $dS.m^{-1}$) resulting from increases in concentration of macro elements (N, P, K, Ca and Mg) and spray before flowering of different hormone levels of 24-epibrassinolide (0, 0.5 and 1 μM) on *C. officinalis* L. seedlings were transferred to 4 L vases containing cocopeat and perlite with a ratio of 50:50 (v/v). The used vases had a polyethylene coating containing 4% anti-UV protection. The daily temperature of the vase was 26±2°C, the night temperature was 19±2°C, the relative humidity 50-60%, and the daytime period 12 h. The base nutrient solution based on Hoagland and Arnon nutrient solution was prepared and its pH adjusted to 6.5 during the pH test; drainage EC of each treatment was controlled every day, and 24-epibrassinolide was sprayed before the first flower emerged. After flowering and at the end of test, developmental parameters such as plant height, number and surfaces of leaves, wet and dry weight of the above- and below-competitions, and number and diameters of flowers were studied.

Determining total phenol of the flower

To determine total phenol, 1 g of the blossomed flowers was weighed, mixed with 6 ml of pure ethanol, and homogenized. Then, 50 μL of floating solution was isolated and diluted with 555 μL of distilled water and 100 μL of Folin-Ciocalteau agent (v:v 1/1). After 5 min at room temperature, 750 μL of the sodium hydroxide containing 20 $g.L^{-1}$ of sodium carbonate was added to each sample and then the solutions were placed at room temperature for 1 h. At the end, the absorption rate of the samples was read at a wavelength of 760 nm. To determine total phenol, the Gallic acid standard was used in concentrations of 250, 200, 150, 100 and 50 $mg.l^{-1}$ and total phenol was determined in terms of $mg.g^{-1}$ (Lemomoine et al., 2007).

Determining the relative water content of leaf

After preparing the leaf samples, disks with diameter of 8 mm were prepared from the middle part of the blade of the leaf. The disks were then weighed with a digital balance (precision of 0.01 g) (FW) and transferred to a petri dish containing distilled water for 3 h in a fridge (4°C). After removing disks from distilled water, their turgid weight (TW) was measured. After determining TW, leaf disks were transferred to the oven (70°C) and their dry weight (DW) was determined. The relative water content of each leaf was calculated using the following relation (Turner, 1981):

$$\mathrm{LRWC} = \frac{\mathrm{DWL-WWL}}{\mathrm{DWL-DWL}} \quad (1)$$

Where,
- LRWC: Leaf relative water content;
- *DWL:* dry weight of the leaf (gr); and
- *WWL:* wet weight of the leaf (gr).

Stability of cellular membrane

In this study, 0.5 g of each treatment was weighed and washed in a petri dish with distilled water. Then, the leaves were crushed and transferred into the falcon tube and 20 ml of distilled water was added to it. The samples were shaken for 30 min with 150 rounds (Amperetable Multitron II, made in Germany), and the initial electrical conduction (EC_1) was read before being transferred to the hot water bath at 95°C. Secondary electrical conduction (EC_2) was read after removing the samples and cooling them, and the electrolyte leakage rate of the samples was calculated as a percentage using the following relation (Lutts et al., 1996):

$$\text{Ion leakage (\%)} = \frac{\mathrm{EC1}}{\mathrm{EC2}} \times 100$$

- IL: Ion leakage (%);
- $EC_{1:}$ Primary Electrical Conduction; and
- $EC_{2:}$ Secondary electrical conductivity.

Measurement of total flavonoid of flower

An amount of 1 g of the dried and powdered plant material was transferred into a hot water bath at 55°C and filtered with Whatman filter paper (GE Healthcare). Ethanol was removed with two filter papers; its water was removed with a rotating operator. Total flavonoid content was determined in the plant extract with aluminum chloride by a colorimetric method and with standard solutions of 12.5, 25, 50, 80 and 100 $mg.l^{-1}$ of Quercetin in methanol 80%. Absorption was measured in a wavelength of 520 nm with blank containing water instead of the sample. The results were mentioned as mg equivalent to Quercetin in each gram of dry weight of the extract (mg of $Quercetin.g^{-1}$) (Basma et al., 2011):

QE= m×V/ M; where
- QE: quercetin equivalents ($mg.ml^{-1}$);
- m: is weight of the dry extract; and
- V: is volume of the extract (ml).

Measurement of the ingredients of C. officinalis L. extract with HPLC

The used column of Synergi MAX80 type with dimensions of 150×4.6 mm, and internal diameter of 4-µm particles (Phenomex USA) was used. The mobile phase included a mixture of pure water and equal ratio of methanol which started moving with gradient, 65% of water and 35% of methanol. The sample was extracted at 50°C with a speed of 1 $ml.min^{-1}$ and injection of 10 ml. Absorption of the extracted solution from a 230-nm column was measured using an ultraviolet detector. The standard of each material was separately used for determination of inhibition time and the unknown samples in the extract (Ganzera et al., 2003).

Statistical analysis of the data

To perform ANOVA and compare means of the measured traits, SAS software version 9.1 was used (SPSS/IBM; New York, United States). Means were compared with Duncan's Multiple Range *test at probability levels of 1% and 5%.*

Conclusion

Considering the results of the brassinosteroids hormone tests, induction of resistance mechanisms in the plant leads to reduction of damage resulting from salinity stress of nutrient solution; 0.5 µM is recommended in *C. officinalis* L. at the second level. The maximum number of flowers was obtained in EC=4.5 $dS.m^{-1}$ and at a hormone concentration of 0.5 µM. As a result, to increase yield of *C. officinalis* L., the best formulation and the best concentration of hormone application were obtained. According to the results obtained from this research, the maximum flavonoid compounds were obtained in EC=4.5 $dS.m^{-1}$ and concentration of 1 µM of hormone. Therefore, a concentration of 0.5 µM in *C. officinalis* L. led to reduction of damage resulting from increase in concentration of the nutrient elements, informing our recommendation of this concentration.

Acknowledgments

The authors would like to thank the Faculty of Agriculture, Islamic Azad University, Damghan, Iran and Islamic Azad University the unit Science and Research, Tehran, Iran for financial support and for providing experimental facilities in this research.

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