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Response of growth, flowering and some biochemical constituents of *Calendula officinalis* L. to foliar application of salicylic acid, ascorbic acid and thiamine

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Abstract

A pot experiment was conducted to evaluate the effect of foliar spray of salicylic acid (0, 50, 100 ppm), ascorbic acid (0, 100, 200 ppm) and thiamine (0, 50, 100 ppm) on vegetative growth, flowering and photosynthetic pigments of marigold (*Calendula officinalis* L.) plants at greenhouse of Shahid Bahonar University of Kerman, Iran. Results showed that application of salicylic acid and thiamine increased number of flowering stems. Stem height increased only by application of thiamine. Fresh and dry weight of the plants affected by all treatments. Foliar spray of all treatments significantly influenced chl.b and total chl contents but differences in the amount of chl.a and carotenoids content was not significant. Application of all treatments reduced stomatal length while number of stomata and electrolyte leakage reduced only by application of salicylic acid. The highest amount of reducing sugars obtained by application of ascorbic acid at 200 ppm and thiamine at 100 ppm, respectively. Application of salicylic acid and thiamine significantly increased the amount of medicinal compound hyperoside.

Introduction

Calendula officinalis L. (Asteraceae) , known as pot Marigold, is an annual plant native to the Mediterranean region (Fonseca et al., 2010). In Iran, Europe and America it is cultivated as an ornamental plant and it's used for medicinal purposes as well. This marigold plant is a source of biological compound which has anti-inflammatory, anti-mutagenic, diuretic, and antispasmodic effects and also can be used in gastro-intestinal, gynecological and eye diseases. Skin injuries also can be treated with marigold (Kumar et al., 2010 ; Re et al., 2009). Pot marigold can be used as a colorant because it primarily contains two classes of pigments, the flavonoids and the carotenoids, which can be used as yellow and orange natural colors, respectively (Khalid & dasilva., 2010). Hyperoside (quercetin-3-O-galactoside) is a medicinal flavonoid compound, which has been shown to possess various biological functions against Reactive Oxygen Species (ROS) induced damage, such as anti-viral activity, anti-inflammatory, antidepressant, hepato-protective and gastric mucosal-protective effects in human (Qin et al., 2010 ; Xing et al., 2011).

Some chemicals were reported as inducer of plant growth and development. In this regard, salicylic acid (SA) is a simple phenolic compound, which plays an important role in regulation of plant growth and development, seed germination, fruit yield, flowering and physiological processes (Klessig & Malamy., 1994 ; Shafiee et al., 2010). SA may be a prerequisite for the synthesis of auxins and/or cytokinins (Metwally et al., 2003). Also photosynthetic rate, stomatal function, respiratory pathways and transpiration could be affected by SA application (Khan et al., 2003). Exogenous application of SA may influence a range of diverse processes in plants, including stomatal closure (Larqué-Saaverda.,1979), ion uptake and transport (Harper & Balke.,1981). Recent studies have indicated that, treating plants with some vitamins resulted in growth improvement. Vitamins could be considered as bio-regulator compound which in relatively low concentrations

exerting profound influences on plant growth regulating factors that in turn influence many physiological processes, such as synthesis of enzymes (Abdel-Halim., 1995 ; Hathout., 1995). Ascorbic acid (Vitamin C) (AsA) is an important metabolite involved in many cellular processes, including cell division (Degara et al., 2003), it is synthesized in higher plants and affects plant growth and development. It is the product of D-glucose metabolism which affects some nutritional cycle's activities in higher plants and plays an important role in electron transport system (El-Kobisy et al., 2005). In soybean plant treated with ascorbic acid, photosynthesis process increased (Golan-Goldhirsh et al., 1995). Shoot and root length, seedling total dry weight in sunflower also increased significantly by ascorbic acid (Dolatabadian & Sanavy., 2008).Talaat (Talaat., 1999) showed that foliar application of ascorbic acid increases the macronutrients content of sweet pepper. Thiamine (Vitamin B_1) is a necessary ingredient for biosynthesis of the coenzyme thiamine pyrophosphate, which plays an important role in carbohydrate metabolism (Robinson.,1973; Hendawy & Ezz EL-Din., 2010). Thiamine (Th) helps to provide an optimal environment for photosynthetic machinery (Jaleel et al., 2006). Th could serve as coenzyme in decarboxylation of α-keto acids, such as pyruvic acid and keto-glutamic acid which has its importance in the metabolism of carbohydrates and fats (Bidwell.,1979). Youssef and Talaat, (2003) reported that pronounced increases in vegetative growth and chemical constituents of rosemary plants by foliar application of thiamine. In this experiment, we have studied the effect of adding SA, AsA and Th to vegetative growth, flowering, physiological, some biochemical and medicinal constituents of marigold plants.

Materials and methods

The experiment was conducted during 2010 at greenhouse of Shahid Bahonar University of Kerman. Plastic pots, 30 cm in diameter, were filled with media containing a mixture of coco-peat and perlite at ratio of 2:1 by volume. Eight seeds were sown in each pot and seedlings were thinned to 3, selecting the most vigorous ones.

At 5 leaf stage, 30 days after planting, marigold plants were sprayed twice with solutions of SA (50 and 100 ppm), AsA (100 and 200 ppm) and Th (50 and 100 ppm). Foliar application of all treatments was carried out two times at 30 days intervals. Untreated plants (control) were sprayed with distilled water. Plants were fertilized using half strength Hogland solution during growth period. The pots were arranged in a Completely Randomize Design (CRD) with 6 treatments and 4 replicates (each replicate contained 3 plants) in addition to the control. Two weeks after second stage of foliar spray and during flowering period, leaf samples were taken from plants and in each photosynthetic pigments (mg/ml), reducing sugars (mg/lit), hyperoside content (%), stomatal number and length (µm), electrolyte leakage(%) were measured. Numbers of flowering stems per plant, plant height (cm), root length (cm), fresh and dry weight of plants (g) were also measured.

- **Stomatal**

Number of leave's stomata was recorded at 0.0234 mm^2 of leaf area. Stomatal length was measured by a micrometer fixed in ocular lens.

- **Electrolyte leakage**

measurement of electrolyte leakage was performed using the method described by Kaya et al (2002). Briefly leaf samples were thoroughly washed with distilled water and transferred into closed vials containing 10 mL deionized water and shake incubated at 25ºC for 6 h. Electrical conductivity (EC) was determined (EC_1) using a Winlab EC-meter. Samples were then transferred into a freezer for 24 h (-22 °C) and EC was measured after melting the samples to liquid state at 25ºC (EC_2). Electrolyte leakage was calculated using the following formula:

$$\mathrm{EC}\ (\%) = \left(\frac{\mathrm{EC}_1}{\mathrm{EC}_2}\right) \times 100$$

- **Pigments**

Chlorophyll (a, b, total), as well as carotenoids content, were determined in fresh leaves using the method described by Lichtenthder (1987). The absorbance of acetone extract of the leaves was measured at 3 wavelengths of (646.8, 663.2 and 470 nm) using spectrophotometer (SPUV-26 SCO-TECH). The concentration of the pigment fraction (chl.a, chl.b, chl.total and carotenoids) was calculated as mg/ml using the following equations:

$$Chlorophyll\ a(\mathrm{mg/ml}) = (12.25\ A_{663.2} - 2.79\ A_{646.8})$$

$$Chlorophyll\ b(\mathrm{mg/ml}) = (21.21\ A_{646.8} - 5.1\ A_{663.2})$$

$$Chlorophyll\ total(\mathrm{mg/ml}) = (chl.a + chl.b)$$

$$Carotenoid(\mathrm{mg/ml}) = {}^{(1000\ A_{470} - 1.8\ Chl.a - 85.02\ Chl.b)}/_{198}$$

- **Reducing sugars**

reducing sugars were performed from leaves extraction by the spectrophotometric method at wavelength of 600 nm recommended by Somogy-Nelson (1952) and glucose concentration (mg/lit) was calculated using the following equation;

$$Y = 0.0216\ X - 0.0097$$

In which X is the reading obtained from spectrophotometer.

- **Hyperoside**

hyperoside content in this study, was obtained from dry petal powder (Ficher.,1981). In this method, obtained data from spectrophotometer at wavelength of 425 were used to calculate the hyperoside amount using the following equation;

$$Hyperoside\ \% = \frac{(1.25 \times E)}{b}$$

in which

E = spectrophotometer data

and;

b = plant sample weight (gr)

All data were subjected to analyze of variance using CRD linear additive model. Treatment means were compared using Duncans multiple range test at 5% of significant. SAS software was used for statistical computation.

Results

Data presented in (table 1 & 4) show that foliar application of SA, AsA and Th significantly increased chl.b and total chl content compared to control plants, but differences existing among plants in terms of chl.a and carotenoid content, were not significant. These results also showed that maximum values of chl.b and total chlorophyll were obtained by application of Th at 100 ppm. The two parameters were increased by 22.75% and 22.99%, respectively. Treatments effect on chl.a and carotenoid content was not significant (table 4). Data presented in table (2 & 5) show that application of SA has significantly reduced electrolyte leakage (%). The lowest percentage of electrolyte leakage was recorded with application of SA at 100 ppm which was 16.52 % lower in comparison to the control plants. Application of Th at 50 ppm increased electrolyte meanwhile, the effect of other treatments on electrolyte leakage was not significant (Table 5). Number of flowering stems was affected significantly by application of SA at 50 ppm and Th at 100 ppm concentration while other treatments' effects on flowering stems were not significant (Table 3 & 6). Table (6) shows that foliar application of Th at 50 and 100 ppm, increases stem height by 33.15 % and 51.83 %, respectively compared to control plants. None of the treatments in this experiment led to significant changes in the root length. Effect of foliar application of AsA and SA on stem height of plants was also not significant (table 6). Application of AsA (100 and 200 ppm) and Th (50 and 100 ppm) increased fresh and dry weight of marigold plants. The highest fresh and dry weights were obtained by application of Th at 100 ppm. Application of Th at 100 ppm increased fresh and dry weight of plants by 22.29 % and 26.7 %, respectively, compared to control plants. Fresh and dry weights of the plants were increased by application of SA at 50 ppm while they were reduced by application of SA at 100 ppm (Table 6). In this study, reducing sugars content was significantly affected by application of all treatments. Generally all treatments increased the amount of reducing sugars. The highest amount was obtained by application of AsA at 200 ppm and Th at 100 ppm which was 27.39 % and 20.14 % higher in comparison to control plants, respectively (table 4). The present results show that application of SA and Th significantly increased the amount of hyperoside while effect of AsA was not significant. The highest hyperoside content was recorded by application of Th at 100 ppm and SA at 50 ppm at which increasing level were 61.4 % and 56.14 % higher compared to control plants, respectively (table 4). Results of this study show that stomata frequency and length are significantly affected by application of all treatments. Generally application of SA reduced the number of stomata per unit of leaf area while stomatal frequency was increased by application of AsA and Th. Lowest number of stomata was recorded at 50 ppm of SA which was 12.32 % lower in comparison to control plants (Table 5). Data, presented in (table 5), show that all treatments reduced stomatal length. Mean stomatal length with application of AsA at 200 ppm was 8.1 micrometer which was 24.6 % smaller in comparison to control plants.

Discussion

Blokhina et al. (2003) believe that AsA has a wide range of important functions such as photo-protection, regulation of photosynthesis and growth in plants. Exogenous application of SA, increased photosynthetic pigments in basil and marjoram (Fatma., 2007). Chlorophyll content of Spirodela plants increases significantly after SA application (Rhoads & McIntosh., 1991). SA also enhances photosynthetic rates of soybean and corn plants (Khan et al., 2003). Photosynthetic pigments in leaves of wheat plants were significantly increased by application of AsA (Amin et al., 2008). Under salt stress condition, application of SA reduces ion leakage in thyme plant (Najafian et al., 2009). Results obtained with application of AsA and Th in this study are not in accordance with the results obtained by El-Hakimi and Al-Ghalibi, (2007) and Korkmaz et al. (2007) who reported that application of AsA and Thpartially retarded leakage of ion in bean and muskmelon plants. Nahed et al. (2009) found that flowering parameters in gladiolus plants were increased by application of Th at 100 ppm. Kord & Hathout (1992) also reported that SA induces flowering in tomato plants. Gamal (2005) and Abd El-Aziz et al, (2007) reported that foliar application

of Th significantly increased all growth parameters of sunflower and syngonium plants. Foliar application of Th also has been shown to increase plant height of *Linum usitatissimum* L. plants (El-Shawy et al., 2008). Application of SA on shoot part growth of soybean plants was significant (Eraslan et al., 2007). Also vegetative growth of *Cupressus sempervirens* L. plants increases significantly by foliar application of AsA (Farahat et al., 2007). El-Tohamy et al. (2008) showed that application of AsA increases fresh weight of eggplant (*Solanum melongena* L.). Similar results were also obtained by Nahed et al. (2007) on *Syngonium podophyllum*. Application of SA at very low concentration (10^{-5}M) stimulated different morphological and growth characteristics of tomato plants while inhibitory effects were observed at low concentrations (10^{-3} M) (Kord & Hathout.,1992). Application of SA increased fresh and dry weight of herbs in basil and marjoram plants (Fatma.,2007).

Tarraf et al, (1999 reported that AsA increases carbohydrate content in lemongrass plant. Nehed et al, (2009) treated gladiolus plants with Th at 100 ppm and observed that total soluble sugar contents had increased in both root and shoot parts. Also, SA application in basil and marjoram plants increases total carbohydrates content (2007). Total flavonoid contents were significantly increased by SA application (Kim et al., 2009). Application of Th, increases total amount of phenols in gladiolus plants (Nahed et al., 2009). Total phenolic compounds in thyme plants were significantly increased by foliar application of Th (Reda et al., 2005). Stomatal function could be affected by SA application (Khan et al.,2003). Application of SA has also been stated that may influence a range of diverse processes in plants, including stomatal closure (Larqué-Saaverda., 1979).

Table 1 . Values of mean squares in the analysis of variance of the data of photosyntetic pigments, reducing sugars and hyperoside content of marigold plants treated by SA, AsA & Th.

	df	Ch.l a (mg. ml^{-1})	Ch.l b (mg. ml^{-1})	Ch.l Total (mg. ml^{-1})	Carotenoid (mg. ml^{-1})	Reducing sugar	Hyperoside %
Treat	6	2.33 ^{ns}	1.2*	4.65*	0.14 ^{ns}	921.6*	0.091*
Error	21	1.90	0.8	1.78	0.16	540.3	0.034
CV %		10.1	19.4	8.97	13.58	8.72	13.45

* and ** are showing the significant effect of the corresponding source of variation at 5 & 1 percent level, respectively. ns means non significant effect.

Table 2 . Values of mean squares in the analysis of variance of the data of physiological parameters in leave of marigold plants treated by SA, AsA & Th.

	df	Stomatal frequency	Stomata length (µm)	Electrolyte leakage %
Treat	6	10.62**	3.47**	65.92**
Error	21	0.52	0.6	7.45
CV %		5.91	8.48	7.19

* and ** are showing the significant effect of the corresponding source of variation at 5 & 1 percent level, respectively. ns means non significant effect.

Table 3 . Values of mean squares in the analysis of variance of the data of vegetative growth and flowering of marigold plants treated by SA, AsA & Th.

	df	Number of flowering stems	Stem height (cm)	Root length (cm)	Plant fresh weight (gr)	Plant dry weight (gr)
Treat	6	4.91*	427.3**	0.12 ^{ns}	160.8*	7.72**
Error	21	2.09	36.1	0.38	44.43	1.15
CV %		13.27	11.31	3.62	11.57	11.35

* and ** are showing the significant effect of the corresponding source of variation at 5 & 1 percent level, respectively. ns means non significant effect.

Table 4 . Mean values of some biochemical characteristics of marigold plants treated with SA, AsA and Th.

	Ch.l a (mg. ml^{-1})	Ch.l b (mg. ml^{-1})	Ch.l Total (mg. ml^{-1})	Carotenoid (mg. ml^{-1})	Reducing sugar (mg/L)	Hyperoside %
Control	8.93 b	4.35 ab	12.96 b	2.78 a	66.9 b	0.57 c
Salicylic acid 50 ppm	10.37 a	4.17 b	14.1 ab	3.1 a	81.08 ab	0.89 ab
Salicylic acid 100 ppm	9.53 b	4.99 ab	14.52 ab	2.79 a	79.19 ab	0.8 ab
Ascorbic acid 100 ppm	10.47 a	4.88 ab	15.34 a	3.07 a	80.21 ab	0.64 bc
Ascorbic acid 200 ppm	10.7 a	4.64 ab	15.34 a	2.98 a	85.23 a	0.69 bc
Thiamine 50 ppm	10.71 a	5.18 ab	15.9 a	3.02 a	81.94 ab	0.88 ab
Thiamine 100 ppm	10.59 a	5.34 a	15.94 a	3.33 a	83.8 a	0.92 a

In each column the differences between the data which are followed by the same alphabet are not significant at 5 percent level.

Table 5. Mean values of some physiological characteristics in leave of marigold plants treated with SA, AsA and Th.

	Stomatal frequency	Stomatal length (µm)	Electrolyte leakage %
Control	11.12 b	10.62 a	38.36 bc
Salicylic acid 50 ppm	9.75 b	8.62 cd	34.52 cd
Salicylic acid 100 ppm	10.75 b	8.5 cd	32.02 d
Ascorbic acid 100 ppm	13.37 a	9.62 abc	38.9 b
Ascorbic acid 200 ppm	13.62 a	8.1 d	39.82 b
Thiamine 50 ppm	13.87 a	10.01 ab	44.8 a
Thiamine 100 ppm	12.87 a	8.87 bcd	37.26 bc

In each column the differences between the data which are followed by the same alphabet are not significant at 5 percent level.

Table 6 . Mean values of some vegetative growth and flowering characters of marigold plants treated with SA, AsA and Th.

	Number of flowering stems	Stem height (cm)	Root length (cm)	Plant fresh weight (gr)	Plant dry weight (gr)
Control	10.25 b	47.5 b	17.1 a	52.25 b	8.66 bc
Salicylic acid 50 ppm	12.65 a	48.12 b	17.06 a	54 b	8.89 bc
Salicylic acid 100 ppm	10.25 b	44.37 b	16.76 a	49.25 b	7.99 c
Ascorbic acid 100 ppm	10.62 b	47.62 b	17.05 a	57.25 ab	8.7 bc
Ascorbic acid 200 ppm	10.5 b	49.37 b	17.21 a	59.25 ab	9.87 b
Thiamine 50 ppm	11.12 b	63.25 a	17.31 a	59.12 ab	10.03 ab
Thiamine 100 ppm	12.8 a	72.12 a	17.21 a	63.9 a	10.98 a

In each column the differences between the data which are followed by the same alphabet are not significant at 5 percent level.

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