



Original Article

Influence of Salicylic Acid and Citric Acid on the Growth, Biochemical Characteristics and Essential Oil Content of Thyme (*Thymus vulgaris* L.)

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Abstract

This study was conducted to determine the effect of foliar salicylic acid (SA) and citric acid (CA) applications on growth, biochemical characteristics and essential oil content of thyme (*Thymus vulgaris* L.) grown under field conditions. Salicylic acid (0.5 and 1 mM) and citric acid (5 and 10 mM) were applied three times during the vegetation at 15 day intervals. Results revealed that foliar application of SA and CA significantly enhanced the vegetative characters *i.e.* fresh and dry weight of thyme as well as pigments (chlorophyll *a* and carotenoids) content and essential oil production (1.3 fold as compared to control). There was a positive correlation between fresh and dry weights, chlorophyll *a* and essential oil. Citric acid treatment especially at 10 mM decreased malondialdehyde (MDA) content. According to our results, applications SA at rate of 0.5-1 or CA at 5 mM should be recommended in order to improve yield and essential oil production in thyme.

Key words: Thyme, Salicylic acid, Citric acid, Growth, Essential oil content

Introduction

Thyme or common thyme (*Thymus vulgaris* L.) is a species of flowering plant in the Lamiaceae family, native to southern Europe from the western Mediterranean to southern Italy. It is a bushy, woody-based evergreen sub shrub with small, highly aromatic, grey-green leaves and clusters of purple or pink flowers in early summer. It is the main source of thyme as an ingredient in cooking and as an herbal medicine [1]. Thyme essential oil also contains a range of additional compounds, such as ρ -cymene, linalool, myrcene, α -pinene, carvacrol, sabinene, 1,8-cineole, α -terpineol, borneol and caryophyllene [2-4]. In medicine, it is used as antispasmodic, antioxidant, antibacterial, antifungal, secretolytic, expectorant, antiseptic, antelmintic and antitusive [5].

The essential oil production depends not only on genetic factors and the developmental stage of plants, but also on environmental factors. It is

desirable to develop techniques of agronomical management to improve essential oil products and their specific compounds. Among other factors influencing plant growth and essential oil production are plant growth regulators. Endogenous levels as well as exogenous application could affect essential oil production and chemical composition [6]. In recent research, plant growth regulators have been shown to improve herb yield in salvia [7], fennel [8], lavender [9], shara [10], ajwain [11], basil and marjoram [12].

In recent years, salicylic acid (SA) has been the focus of intensive research due to its function as an endogenous signal mediating local and systemic plant defense responses against pathogens and abiotic stresses such as drought, chilling, heavy metal toxicity, heat, and osmotic stress. Besides this function during biotic and abiotic stresses, SA plays a crucial role in the regulation of physiological and biochemical processes during the entire lifespan of the plant [13]. Citric acid (CA) is

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a natural product of citrus fruits such as lemons, limes, oranges and tangerines [14]. Some studies show that citric acid, when used in smaller doses may be beneficial to plants. Thus, the objective of current study was to investigate the effect of SA and CA on growth parameters, photosynthetic pigments and essential oil content of thyme plants.

Material and Methods

Plant Materials and Experimental Design

This experiment was conducted at the research field of Islamic Azad University, Karaj Branch, Karaj (35°45' N, 51°56' E, 1313 m above the sea level), Iran. There is characterized by sunny, hot and dry days during summer seasons. The field experiment was set up in a complete randomized block design (CRBD) with five treatments and six replications. Seeds of thyme (*Thymus vulgaris* L.) were sown in trays filled with coco peat in mid February 2011 in a controlled environment greenhouse. Plantlets of uniform height were selected and transplanted to field in lines in distance of 50×20 cm on 9 April. Physical and chemical properties of the soil were evaluated up to 30 cm depth (Table 1). Foliar spray of SA (0.5 and 1 mM) and CA (5 and 10 mM) were made with a hand sprayer on field grown plants three times, at 15 day intervals. Distilled water sprayed plants were considered as control. Solutions were sprayed to all plant surfaces to the point of runoff. Standard inter-cultivation practices like irrigation, weeding and manuring were followed during the entire crop development. Plants at flowering stage (11 November) were cut 10 cm above the soil surface. Determination of fresh and dry weight, and photosynthetic pigments

Dry weights of plants were determined after drying the fresh plant organs in electrical oven at 70 °C for 48 hours according to Schafleitner [15].

Chlorophyll *a* and carotenoids were determined according to Wettstein [16] method. 2 g of fresh leaves of each sample was ground with a small amount of acetone 85% (v/v) until the mixture became homogeneous, then the extract was filtered

and the filtrate was collected in a flask and completed to 100 ml with acetone 85% (v/v). The amounts of chlorophyll *a* and carotenoids were determined spectrophotometrically by reading the absorbance at 665, 649 and 470 nm and calculated according to the following equations using acetone 85% (v/v) as a blank.

$$\text{Chlorophyll } a = 10.3 A_{663} - 0.918 A_{644}$$

$$\text{Chlorophyll } b = 19.7 A_{644} - 3.87 A_{663}$$

$$\text{Carotenoids} = 4.2A_{440} - [0.0264 \text{ Chl.}(a) + 0.426 \text{ Chl.}(b)]$$

The pigment content are expressed as unit's mg per gram-fresh weight (mg·g⁻¹ FW).

Determination of Essential oil Content

Essential oil was extracted from harvested fresh plant material using a steam distillation apparatus. The oil fraction was recovered from the water phase using petroleum ether.

Malondialdehyde (MDA) Assay

Malondialdehyde (MDA) was determined according to the method described by Hodges *et al* [17] and was calculated according to Heath and Pacher [18]. 100 mg of leaf samples was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA), and centrifuged at 10000 g for 5 min at 4 °C. Aliquot of 0.3 ml supernatant was mixed with 1.2 ml of 0.5% (w/v) thiobarbituric acid (TBA) prepared in TCA 20% (w/v), and incubated at 95 °C for 30 min. The solutions then were cooled in an ice bath for 5 min, and centrifuged at 10000 g for 10 min at 25 °C. The supernatant absorbance at 532 nm was measured using a spectrophotometer. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM⁻¹.cm⁻¹.

Statistical Analysis

The data were analyzed statistically by analysis of variance (ANOVA) using SPSS.16 statistical software. Means were compared using Duncan's Multiple Range Test (DMRT) at 5% probability level. Relationships among variables were determined based on Pearson's correlations coefficient test at 1 and 5% probability level.

Table 1 Physical and chemical characteristics of experimental field soil.

Depth (cm)	Physico-chemical characteristics										
	pH	EC (dS.m ⁻¹)	Organic matter (%)	CaCO ₃ (%)	N total (%)	P (ppm)	K (ppm)	Zn (ppm)	Sand (%)	Silt (%)	Clay (%)
0-30	7.9	3.4	1.6	6.2	17	10.6	250.2	1	5	35	60

Results and Discussion

Fresh and dry weight

Fresh and dry weights were significantly enhanced by exogenous application of salicylic acid (SA) and citric acid (CA) as compared to the unsprayed plants (Table 2). These promoting effects were at maximal (108 and 109 g.plant⁻¹) at 1 mM SA and 10 mM CA for fresh weight parameter, but the highest dry weight (42 and 43 g.plant⁻¹) was noted at 0.5 mM SA and 5 and 10 mM CA and that in control they were 87 and 33 g.plant⁻¹, respectively. Same results for the positive effects of SA on fresh and dry weight have been reported by Gharib [12] in basil and marjoram, Khandaker *et al* [19] in red amaranth and Abdou and Mohamed [20] in mint. Similarly, in other studies SA has increased plant growth and development in the stressed thyme [21], shara [10], coriandrum [22] and peanut [23]. On the other hand, a Pearson correlation coefficient revealed that there is a positive and significant correlation between Chlorophyll *a* and fresh ($r=0.56$) and dry ($r=0.52$) weights (Table 3). It seems that SA and CA by increasing the amount of chlorophyll *a* (Table 2), caused more efficient in photosynthesis which ultimately leads to increasing fresh and dry weights. This is consistent with the statement of Rivas-San Vicente and Plasencia [13], who suggested that the growth promoting effects of SA could be related to changes in the hormonal status or by improvement of photosynthesis, transpiration, and stomata conductance. Also, Maleki *et al* [24] reported the enhanced growth attributes (shoot and root fresh and dry weights) with foliar application of CA in sweet basil. El-Tohamy *et al* [25] found that CA treatment resulted in improvement of vegetative growth parameters of bean, expressed as fresh weight of plants and branches number per plant in relation to water stress. In addition, the authors indicated that the improvement of growth and quality of drought-stressed bean plants in response to CA application can be explained by the enhancement of water status during drought stress due to osmotic adjustment. On the other hand, it plays an important role in the mitochondrial CA cycle. The CA cycle involves the biosynthesis of several organic acids, many of which serve as precursors for the biosynthesis of several groups of amino acids [26]. This can ultimately enhances yield and plants fresh and dry weights.

Chlorophyll *a* and carotenoid content

Chlorophyll content is an indicator of leaf photosynthesis and the major role of the carotenoids is to act as light receptors and protectors of the photosynthetic apparatus [14, 27]. As shown in Table 2, foliar spray of SA (1 mM) and CA (5 mM) increased chlorophyll *a*. Among the treatments, only the application of CA at 5 mM improved the carotenoids pigments over the control. SA at 1 mM also increased the carotenoids content, but this increase was not significant. This agrees with the findings of Gharib [12], Khandaker *et al* [19], Jalal *et al* [10], El-Tohamy *et al* [25] and Kong *et al* [23], that reported SA or CA treatment had a positive effect on photosynthetic pigments and plants sprayed with them showed a higher total chlorophyll content compared to control. Song *et al* [28] studied the effects of oxalic acid and CA solutions on chlorophyll contents and photosynthesis of *Fraxinus mandshurica* Rupr. seedlings and found that organic acids of appropriate concentrations increased chlorophyll contents, chlorophyll *a* and *b* contents varied more than carotenoid content. They concluded that effects of different organic acids on chlorophyll contents and photosynthesis followed the series: CA > oxalic acid and it might be relative to chemical structure, dissociation constants and organic ligand-metal stabilities of two kinds of organic acids. Czerpak *et al* [29] also found that SA strongly stimulated action on the content of chlorophylls *a* and *b* and carotenoids in *Wolffia arrhiza* (L.) Horkel ex Wimm. Recent evidence suggests that SA is an important regulator of photosynthesis because it affects leaf and chloroplast structure, stomata closure and chlorophyll and carotenoid contents [13].

Essential oil content

The mean values of essential oil content per plant increased 31.3, 36.4 and 29.8% (5.8, 6.0 and 5.7 ml.plant⁻¹) over control plants by application of SA at 0.5 mM and CA at 5 and 10 mM, respectively (Table 2). Ozguven and Tansi [26] reported essential oil content and its components in thyme were significantly affected by both climatic and ecological conditions. Moreover, several factors such as plant growth regulators influence secondary metabolites quantity and quality of medicinal and aromatic plants [9]. Abdou and Mohamed [20] revealed that treated mint plants with SA + ascorbic acid increased essential oil concentration by 87% compared with the NPK-fertilized plants treated with tap water.

Table 2 Effect of salicylic acid (SA) and citric acid (CA) on growth, biochemical characteristics and essential oil content of thyme plants

Treatment (mM)	Fresh weight (kg.m ⁻²)	Dry weight (kg.m ⁻²)	Essential oil (ml.m ⁻²)	Chlorophyll <i>a</i> (mg.g ⁻¹)	Carotenoids (mg.g ⁻¹)	MDA (nM.g ⁻¹)
control	0.87 b	0.33 b	44.21 b	0.60 b	0.108 b	0.491 a
SA 0.5	1.02 ab	0.43 a	58.07 a	0.74 ab	0.104 b	0.367 bc
SA 1	1.08 a	0.40 ab	55.06 ab	0.89 a	0.129 ab	0.469 a
CA 5	1.03 ab	0.42 a	60.32 a	0.89 a	0.136 a	0.406 b
CA 10	1.09 a	0.42 a	57.42 a	0.66 b	0.101 b	0.341 c
p-value	0.041	0.013	0.025	0.031	0.064	0.002

Different letters within columns indicate significant differences ($p < 0.05$).

Table 3 Pearson correlation coefficient of evaluated traits of thyme.

Trait	Fresh weight	Dry weight	Essential oil	Chlorophyll <i>a</i>	Carotenoids
Dry weight	0.84 **				
Essential oil	0.82 **	0.97 **			
Chlorophyll <i>a</i>	0.56 *	0.52 *	0.63 *		
Carotenoids	0.20 ^{ns}	0.11 ^{ns}	0.30 ^{ns}	0.87 **	
MDA	-0.59 *	-0.80 *	-0.73 *	0.06 ^{ns}	0.39 ^{ns}

ns, * and **: no significant and significant at 5 and 1% probability level, respectively.

Also, Gharib [12] found that oil percentage and yield per plant increased 5.49 and 26.66%, respectively by SA application at 10^{-3} M in basil. He concluded that the increment in oil yield might be due to the increase in vegetative growth, nutrients uptake or changes in leaf oil gland population and monoterpenes biosynthesis. This is in agreement with the statement of Hassanpour Aghdam *et al* [9], who showed that the elevated leaves fresh and dry weight of French lavender plants under 300 mg l^{-1} GA₃ application likely led to the suitable interactions of primary and secondary metabolism in favor of essential oil production. The positive correlation results between essential oil and fresh ($r = 0.82$) and dry ($r = 0.97$) weight (Table 3) also confirm this hypothesis. Jaafari and Hadavi [30] indicated that both CA and SA improved the essential oil yield of basil. In another experiment, they showed that combination of CA and malic acid increased wet and dry weight and essential oil yield of dill (*Anethum graveolens* L.) comparing with control treatment [31]. They stated that CA had tended the internal metabolism towards a higher carbon flow to secondary metabolism pathways. The effect of citrate on the yield is another finding, which is responsible for the increase in the essential oil yield as well. Overall, their findings suggest that this effect is in part due to an increase in the proton pumping rate in roots being supported or induced by organic acids supplied via foliar sprays.

MDA content

The MDA content was reduced 17.3 to 30.5% by SA (at low concentration) and CA when compared

to the control (Table 2). SA at high concentration did not cause a decrease in the MDA content (0.469 and 0.491 nmol.g^{-1} FW in 1 mM SA and control, respectively). The level of lipid peroxidation has been widely used as an indicator of free radical mediated damage to cell membranes under stressful conditions. MDA is one of the final products of the peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage [4]. Thus, reducing MDA in SA and CA treatments is indicating their role in an increase in stress tolerance. Sun *et al* [32] indicated that exogenous SA (less than 0.25 mM) enhanced drought tolerance of barley leaves, decreasing the electron leakage and MDA content. Sun and Hong [33] also showed that plant growth was significantly improved by CA application during saline stress conditions. According to authors, CA seems to be an important component of the stress response in *Leymus chinensis* (Trin.) Tzvelev. There was a significant negative correlation between MDA and fresh ($r = -0.59$) and dry ($r = -0.80$) weights and essential oil ($r = -0.73$) (Table 3). Therefore, we propose that these organic acids with increasing stress tolerance led to improved plant growth characteristics.

Conclusions

Since this experiment was conducted in field conditions, plants are likely to have been exposed to stress and it seem to that SA and CA applied on thyme stimulated the growth and oil content by

enhancing photosynthesis and more tolerance to stress. As far as we know, this is the first study linking increased growth of thyme by CA. However, further investigations are required to elucidate the possible role of these on plant growth regulating activity.

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