Growth, essential oil yield and components of summer savory (*Satureja hortensis* L.) influenced by Salicylic acid and Proline

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Abstract

Today, the use of essential oils of medicinal plants has become very important for human health. Proper nutrition of medicinal plants plays a critical role in the quantitative and qualitative performance of their essential oils. In order to investigate the effects of foliar application of salicylic acid and proline on the vegetative and biochemical characteristics of savory, an experiment was performed in a randomized complete block design with three replicates at the Research Farm in Islamic Azad University, Saveh branch, Iran. Treatments included salicylic acid (0.5 and 1 mM) and amino acid proline (1.5 and 3 g/L), and the control. Foliar application was done in three phases: stem elongation, flower initiation, and flowering. Plant height, number of flowers and side-stems, fresh and dry weights of leaves and shoots, and essential oil percentage and ingredients were investigated. Results indicated that the maximum number of flowers (21.66), plant height (47.26 cm), leaf dry weight (5.34 g), and leaf fresh weight (16.17 g) were observed by applying 1.5 g/L proline. The maximum shoot dry weight (8.85 g), shoot fresh weight (48.53 g), and number of side-stems were observed by applying 1 mM salicylic acid. The highest (183.65 kg/ha) and the lowest (76.92 kg/ha) essential oil yield were achieved by applying 1.5 g/L proline and control, respectively. The most typical components of essential oil were γ-terpinene and Carvacrol. In conclusion, the highest effects on essential oil yield were obtained under the foliar application of 1 mM salicylic acid and 1.5 g/L proline.

Keywords: α-terpinene,Carvacrol, foliar application,proline,γ-terpinene

Poorghadir M., Mirjalili SA., Mohammadi Torkashvand A., Moradi P.2021. 'Growth, essential oil yield, and components of summer savory (*Satureja hortensis* L.) influenced by two biostimulants'.*Iranian Journal of Plant Physiology*11(5): 3863-3872.

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Received: December, 2020
Accepted: August, 2021

Introduction

The side effects of chemical medicines and the desire for health improvement by more natural products have drawn many attentions to medicinal plants and their production (Van Wykand Wink, 2017). Summer Savory (*Satureja hortensis* L.) is an annual aromatic herb, 30-60 cm tall, with slender, erect, and hairy stems. Leaves are opposite, sessile or sub sessile, and linear to linear-oblanceolate, with many little glandes, rich in essences, on their surface. There are uppermost axillary inflorescences, with 2-17 from white to purple flowers arranged in whorls (Kataret al., 2017). Kashaniet al. (2018) reported that savory is useful in treating muscle pains or myalgia, muscle cramps, nausea, diarrhea, and infectious diseases; in addition, these plants are digestive-friendly, diuretic, painkiller, anticancer, expectorant, and stimulant, and are also used as stomach tonic. These effects are attributed to the presence of Carvacrol in savory essential oil, which is an active pharmaceutical ingredient (Kremer et al., 2015). Kataret al. (2017) introduced Carvacrol, γ-terpinene, α-terpinene, and p-cymene as main constituents of this plant. The increased amounts of these compounds improve the qualitative characteristics of savory plants and improve their efficiency in treating diseases.

Considering the importance of extending sustainable agriculture and using non-harmful medicinal plants, it is critical to use the appropriate materials and methods to increase essential oil and active ingredients in medicinal plants (Kapoor et al., 2018; Mohammadi et al., 2019). To this end, nowadays, various plant regulators are applied to improve qualitative and quantitative growth of medicinal plants. Salicylic acid is one of the most frequently used growth-regulators which affects various plant processes including seed germination (Anaya et al., 2018), stomata opening (Belt et al., 2017), ion adsorption and transport (Talaat, and Laila, 2010), membrane permeability (Nimiret al., 2016) and growth rate (Khan et al., 2015). Salicylic acid is considered as a growth-regulator which depends on its concentration, plant type, growth phase, and environmental conditions. It plays an important role in regulating physiological processes including growth, ion adsorption, photosynthesis, germination, and essential oil expression in medicinal plants (Khalil et al., 2018; Anaya et al., 2018). In addition, salicylic acid is considered as an important molecular signal in plant fluctuations in response to environmental stresses (Khan *et al.,* 2015). The findings of Faraji-Mehmanyet al. (2016) illustrated that spraying summer savory (*Satureja hortensis*) plants with 1, 2, and 3 mM salicylic acid improved morphological, physiological, and biochemical characteristics of the plant. They suggested salicylic acid as a plant growth regulation agent for improving essential oil yield and content. Furthermore, Abrunet al. (2016) found that application of 1 mM salicylic acid can increase stem diameter, leaf thickness, total flavonoid content, total phenol content, and antioxidant activity in savory plant compared to control.

Amino acids act as stimulant compounds for qualitative and quantitative growth of plants (Hildebrandt et al., 2015). These compounds have important roles in biosynthesis of hormonal and secondary metabolites. Generally, amino acids are materials with stimulating effect on metabolism and metabolic processes for improving plant efficiency (Hildebrandt et al., 2015). Therefore, application of biostimulants can be highly important for successful production of a medicinal plant (Kapoor, 2017). Due to the presence of amino acids in combination and formulation of biostimulants, they lead to 2.5 times mRNA increase in transcription, activation of the hormones affecting vegetative growth, activation of carbohydrate formation process, increase of adsorption and transportation of nutrients, and improvement of plant protein content. They improve quantitative and qualitative characteristics, especially essential oils, under environmental stress condition, and thereby affect the quantitative and qualitative indices of the plants (Singh, 2015; Kapoor, 2017). Improvement of physiological indices and main components of essential oil including γ-terpinene, carvacrol, and p-cymene are the positive effects of amino acids reported in savory (El-Gohary*et*al., 2015).

The aim of the present study was to investigate the effects of foliar application of salicylic acid and amino acid proline on the quantitative and qualitative characteristics of the medicinal plant, savory. The traits investigated included yield, efficiency, and main components of essential oils such as γ-terpinene, carvacrol, α-pinene, and p-cymene.

Materials and Methods

The study was arranged based on a randomized complete block design with three replicates at the Research Farm of Islamic Azad University, Saveh Branch in 2018-2019. The experiment included treatments with two salicylic acid levels (0.5 and 1 mM) and two proline levels (1.5 and 3 g/L) and a control.

Table 1
Physiochemical properties of the soil

Sand	Silt	Clay	Texture	Available potassium	*EC*	pH	Total nitrogen	Organic carbon
%	%	%	-	mg/kg	dS/m	-	%	%
37	37.6	25.4	Loam	560	1.43	7.3	0.09	1.33

Soil analysis was performed before starting the study process in order to determine thephysicochemical properties of the soil used in the study(Table 1).

Plant materials

Seeds of annual summer savory were supplied form PakanBazr™ Company, Isfahan. Plots were prepared in 2.5×2 m dimensions and 1m spacing between them. Ridges and furrows were created on the farm and seeds were planted manually in June 2018. The rows were 50 cm apart from each other and the distance between plants on a row was 20 cm. The first irrigation was performed immediately after planting and considering weather conditions, irrigation was repeated every three days up to the harvest time. The thinning practice was done at 4-6 leaf stage.

Treatments

Germination took place 7 days after planting. The plantlets entered the 4-leaf stage12 days after germination.The first step of spraying (foliar application) was performed21 days after germination, which was coincident with stemming. The next steps of spraying were carried out 14 and 21 days later at the beginning of plant blossoming and flowering stages, respectively. Weeding practice was carried out during the plant growth season when it was considered necessary.

Plant sampling and data collection

Morphological characteristics including plant height and stem diameter were measured at early flowering stage. At the flowering stage, when the essential oil has the highest amount and quality, plants were collected to measure morphological and physiological characteristics, essential oil content, and some properties including fresh and dry weights of shoots, fresh and dry weights of leaves, number of flowers, total chlorophyll, and essential oil percentage. Then, the plants were shade-dried for two weeks before they were milled. Finally, the essential oil was extracted by steam distillation using a Clevenger apparatus for three hours, the essential oil percentage was measured and its yield was calculated using Eq.1 as below:

Essential oil yield= Essential oil content × grain yield (1)

The spectrophotometry method (UV/Vis T90 Company PG) was used to measure chlorophyll a and chlorophyll b at wavelengths of 646.2 and 663.2 nm, respectively. Chlorophyll a, b, and total chlorophyll contents were calculated using Eqs.2 and 3, and 4, respectively (Porra et al., 1989):

$$\text{Chl a (mg/ml)} = 12.25\,A_{663} - 2.79\,A_{645} \quad (2)$$

$$\text{Chl b (mg/ml)} = 21.50\,A_{645} - 5.10\,A_{663} \quad (3)$$

$$\text{Chl T(mg/ml)} = 20.2\,A_{645} + 8.02\,A_{663} \quad (4)$$

Component analysis

Table 2
The effects of spraying salicylic acid and amino acid proline on growth, physiological characteristics, and qualitative and quantitative yield of summer savory

treatment	Plant height	Stem diameter	Branch number	Shoot fresh weight	Shoot dry weight	Leaf fresh weight	Leaf dry weight	Number of flower	Total chlorophyll
	(cm)	(mm)	-	(g)	(g)	(g)	(g)	-	(mg/g Fw)
Control	39.33±3.71b	1.66±0.18b	18.66±2.25c	28.73±3.35d	5.02±0.56d	9.91±1.12d	2.55±0.28d	13.66±2.52d	0.39±0.03d
Salicylic Acid (0.5 mM)	40.46±3.84b	1.76±0.14ab	20±4.16b	32.26±2.76c	6.14±0.94c	10.75±1.88d	2.76±0.47d	15±3.61c	0.44±0.02 c
Salicylic Acid (0.5 mM)	39.4±4.27b	2.13±0.15ab	25.66±3.21a	48.53±3.31a	8.85±0.55a	12.12±0,89c	3.12±0.31c	16.66±2.26c	0.48±0.04bc
Proline (1.5 g/l)	47.26±3.4a	2.54±0.12a	20.66±3.21b	39.34±3.31b	6.83±0.52b	16.17±1.1a	5.34±0.28a	21.66±3.21a	0.66±0.08a
Proline (3 g/l)	41.2±4.79b	1.95±0.19ab	20±3.0b	35.26±2.22c	6.64±0.37c	13.75±0.74b	4.02±0.18b	18±3.0b	0.56±0.02b

Numbers followed by the same letter are not significantly different (p<0.05).

A GC/MS apparatus was employed to determine the essential oil components including γ-terpinene, carvacrol, α-pinene and p-cymene.The gas chromatograph device was an Agilent 6890HP-5MSwith a 30-m-long column, inner diameter of 0.25 mm, and layer thickness of 0.25 µm. Temperature schedule of the column was set as follows: oven initial temperature of 50 ℃kept for5 minutes, temperature gradient of 3 ℃ per minute, temperature rise to 240 ℃ with the rate of 15 ℃/min, and temperature rise to 300 ℃ kept for 3 minutes. Temperature in injection chamber was 290 ℃ and helium was used as a carrier gas with 0.8 mm/min flow rate. A 5973Agilent mass spectrometer with an ionization voltage of 70 electron-volts was used and the temperature in ionization source was 220 ℃. In all spectra of GC/MS, the Kovats index for every peak was calculated using exit pattern of normal alkanes and spectral inhibition index. Then, by comparing the measured Kovats index and its relevant spectrum with references and also by face to face comparing the spectra with information in Wiley 275 e-library, Adams (2007), and other references, the spectra for each object were interpreted and their essential oil components and chemical formula were identified.

Results

Based on the obtained results, the effects of foliar application of salicylic acid and proline on most studied characteristics including plant height, stem diameter, number of branches (side-stems), fresh and dry weights of shoots and leaves, number of flowers, total chlorophyll content, essential oil percentage, and yield of summer savory plants were statistically significant at p≤0.01. Mean comparison indicated clear differences among treatments and the effects of spraying 0.5 and 1 mM salicylic acid solution and 1.5 and 3 g/L amino acid proline on growth, physiological characteristics, and quantitative and qualitative performances of summer savory (Table 2).

Treating plants with proline and salicylic acid resulted in taller plants. The maximum height of plants was 47.27 cm in the treatment with 1.5 g/L amino acid proline which was significantly different from other treatments. The shortest plantswere observed in control with 39.33 cm plant height which was not significantly different from the treatments with salicylic acid and 3 g/L proline (Table 2).

Treatments with proline and salicylic acid increased stem diameter. However, the highest stem diameter of 2.65 mm was obtained in the treatment with 1.5 g/L proline, which was significantly different from stem diameter

Table 3
The effect of salicylic acid and Proline on essential oil components (%) of summer savory

Treatment	α-pinene	γ-terpinene	ρ-cymene	Carvacrol
Control	6.88±0.27 a	21.33±1.14 d	5.06±0.24 d	28.45±2.04 d
Salicylic Acid(0.5 mM)	5.06±0.15 d	24.36±1.05 c	5.63±0.32 c	28.91±2.28 d
Salicylic Acid(1 mM)	5.33±0.11 c	25.66±1.31 b	6.26±0.23 b	31.93±1.85 b
Proline(1.5 g/L)	5.83±0.84 b	27.80±1.27 a	7.43±0.68 a	34.00±1.41 a
Proline (3 g/L)	5.03±0.12 d	24.40±0.92 c	5.63±0.32 c	29.03±2.52 c

Numbers followed by the same letter are not significantly different (p.<0.05)

obtained in other treatments. The treatments with 0.5 and 1 g/L salicylic acid and 3 g/L proline were not significantly different from control and the treatment with 1.5 g/L proline. The minimum stem diameter (1.66 mm) was obtained in control.

Treatments with proline and salicylic acid increased number of flowers per plant. Applying 1.5 g/Lprolineled to 58% increase in the number of flowers (Table 2). Numbers of flowers under 0.5 and 1 mM salicylic acid treatment were 15 and 16.66 flower per plant, respectively, showing no significant difference. The lowest number of flowers per plant (13.66 flower/plant) was observed in control showinga significant difference from in application of all other treatments.

Moreover, in comparison to control, applying 1.5 g/L proline increased fresh and dry weights of plant leaves by 63% and 100%, respectively (Table 2).

Treatments with proline and salicylic acid increased the number of side-stems.The highest number of side-stems (25.66) was obtained in the treatment with 1mM salicylic acid, which was significantly different from the results obtained in control and other treatments (Table 2). Also, there was a significant difference between treatment with 0.5 mM salicylic acid and treatment with 3 g/Lproline. The lowest number of side-stems (18.66) was recorded in control plants, showing significant difference from other treatments.

The highest freshand dry weights of shoots (48.53 and 8.85 g,respectively) were obtained in treatment with 1mM salicylic acid marking a significant difference from the other treatments of the study. The lowest fresh and dry weights of shoots were 28.73 g and 5.02g, respectively,

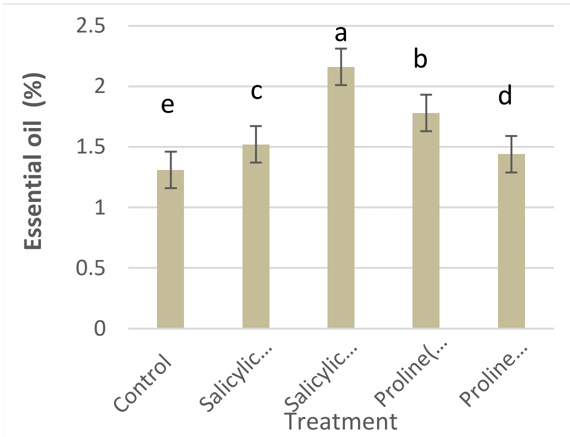

Fig. I.The effect of foliar application of salicylic acid and proline on the essential oilpercentage of summer savory

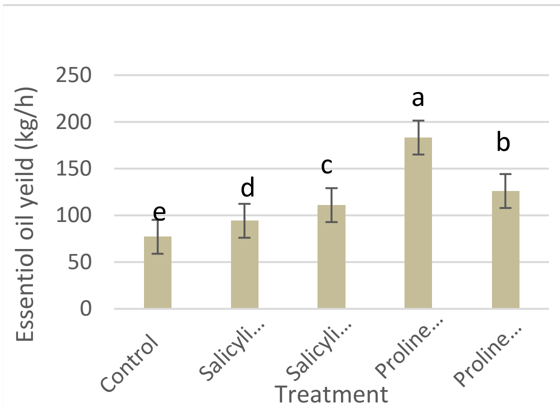

Fig. II. The effect of foliar application of salicylic acid and proline on the essential oil yield of summer savory

recorded in control plants showing a significant difference from the other treatments (Table 2).

Treatments with proline and salicylic acid increased plant chlorophyll content.The highest chlorophyll content was 0.66 mg/gfreshleaf in the treatment with 1.5 g/L proline, and it was significantly different from the control and other treatments (Table 2). The lowest chlorophyll content of 0.39 mg/gfreshleaf was observed in control showing a significant difference from all treatment levels. Using 1 mM salicylic acid led to 23% increase in chlorophyll contentcompared to the control.

The highest percentage of essential oil (2.16%) was achieved using 1 mM salicylic acid marking a significant difference from the control and other treatments of the study (Fig. I). Compared to control, applying 0.5 and 1 mM salicylic acid and 1.5 and 3 g/L amino acid proline led to 46%, 67%, 36%, and 7% increase in essential oil percentage, respectively. Treatments with proline and salicylic acid increased the essential oil yield of the plants under study. The highest essential oil yield was 183.09 kg/ha under the treatment with 1.5g/L proline, which was significantly different fromthe other treatments and control (Fig. II). The lowest essential oil yield (76.92 kg/ha) was obtained from the control plants.

According to the results presented in Table 3, carvacrol and α-pinene were the most typical components of plant essential oil. The highest Carvacrol amount (34%) was observed in the treatment with 1.5 g/Lproline, showing a significant difference from treatments with salicylic acid and other proline concentrations. As can be seen in Table 3, the highest amounts of γ-terpinene (8.27%), α-pinene (83.5%) and ρ-cymene (43.7%) were obtained in the treatment with 1.5 g/Lproline, showing a significant difference from the treatments with salicylic acid and other concentrations of proline.

Discussion

Findings of the present study suggested that foliar application of salicylic acid and proline significantly improved most studied characteristics of the summer savory plants under study. The positive effects of proline could be explained by the various roles of amino acids in plants. Amino acids are building blocks of proteins as well as precursors for a wide range of other molecules that serve important functions in plants. Amino acids are involved not only in the synthesis of compounds such as protein, amines, alkaloids, vitamins, enzymes, terpenoids, and plant hormones that control various plant processes, but also are crucial to stimulating cell growth and act as buffers, provide a source of carbon and energy, and protect the cells from ammonia toxicity, with amide formation (Wafaa et al., 2021).

The maximum and minimum plant heights were recorded in the treatment with 1.5 g/L amino acid proline and control, respectively. These results are consistent with the findings of previous studies (Mohammed and Al-Saad, 2019). In fact, spraying proline solution was reported to result in production of biosynthetic enzymes, improving vegetative growth in the plant through increasing internode length, and eventually leading to taller bushes (Kapoor, 2017; Singh, 2015). Fallahi et al. (2018) found that various concentrations of growth-regulators, including thiamine, increased the vegetative growth of basil plants. Mahjoub et al. (2011) suggested that applying different concentrations of putrescine significantly improved the height of Dahlia plants.

The maximum and minimum stem diameters in the study were recorded for the plants treated with 1.5 g/L proline and control, respectively. This is consistent with the results reported by Fallahi et al. (2018). Nahed et al. (2009) attributed the increased stem diameter of the plants under foliar application of amino acid proline to its impact on increasing cell division.

Results showed that treatments with proline and salicylic acid increased number of flowers per plant. Akhtar et al. (2016) found that amino acid application resulted in more flowers in rose plants. This can be attributed to the effect of amino acids on plant physiology, synthesis of metabolites, plant growth, and flowering (Slocum and Galston, 1985).

Talaat and Laila (2010) studied the effects of putrescine and trans-cinnamic acid on basil plant and found that the treatments increased shoot yield in the first and second shoot harvesting. They concluded that applying 150 mg/L putrescine (highest level) had the most significant effect on the growth indices of basil including plant height, and number of leaves per plant. Teixeira et al. (2017) suggested that application of amino acids, including glutamate, phenylalanine, cysteine, and glycine increased the number of leaves in soybean. Aghaei et al. (2019) pointed that the application of organic/biological fertilizers along with L-phenylalanine spraying had beneficial and effective role in improving the growth characteristics, shoot performance, photosynthetic pigments, and active substances of hyssop (*Hyssopus officinalis*).The effect of proline on plant growth and number of leaves can be due to its ant-oxidative characteristics, helping cation-anion balance, and highlighting its probable role as a nitrogen source (Singh, 2015).

Treatments with proline and salicylic acid increased the number of side-stems and the dry and fresh shoots of summer savory plants in this study. These findings are consistent with those reported by Faraji-Mehmanyet al. (2016), Abrun et al. (2016), and Ghaderiet al. (2015) who studied the effect of spraying 1 mM salicylic acid on summer savory (*Satureja hortensis* L.). The increase can be attributed to the fact that stomatal conductance determines CO_2 entrance rate and thus, determines the production of assimilates and photosynthesis products (Anaya et al., 2018; Belt et al., 2017; Khalil et al., 2018; Talaat and Laila, 2010).Foliar application of salicylic acid and amino acid proline, through increasing the opening degree of stomata, improves growth rate and photosynthesis, eventually leading to an increase in shoot yield (Khan et al., 2015; Belt et al., 2017).

In this study, foliar application of proline and salicylic acid increased plant chlorophyll content. Teixeira et al. (2017) reported that application of glutamate, phenylalanine, cysteine, and glycine amino acids increased soybean chlorophyll content. In addition, Sh-Sadak et al. (2015) indicated that amino acids have positive effects on the amount of photosynthetic pigments of broad/fava bean. Talaat (2005) suggested that foliar application of putrescine and tryptophan amino acids in *Carthamus roseus* L. increased growth, photosynthetic pigments (including chlorophyll a and b, and carotenoids), soluble and insoluble sugars, protein, and alkaloids. In another study, Ghassemi-Golezani et al. (2015) reported that foliar application of salicylic acid increased chlorophyll content in Mung bean. The effect of salicylic acid has also been reported on chlorophyll content of *Carum copticum* L. plants (Ghassemiet al., 2017).

The essential oil yields of summer savory plants were positively affected by foliar application of proline and salicylic acid in this study. Since essential oils are terpenoidal compounds and the blocks (isonoides) such as Isopentenyl pyrophosphate and Dimethylallyl pyrophosphate need ATP and NADPH to be built and considering the importance of nutrients in formation of these compounds, it seems that foliar application by making nutrients available, increasing the adsorption of nutrients including nitrogen, as well as synthesis of sugars and carbohydrates increased the essential oil yield of this medicinal plant (Rahimzadehet al., 2011). Many studies confirm the findings of this study showing the positive effects of proline and salicylic acid on the essential oil yields of various plants. Ghassemiet al. (2017) found that spraying salicylic acid increased essential oil percentage in *Carum copticum* L. Faraji-Mehmanyet al. (2016) reported that spraying summer savory (*Satureja hortensis* L.) shoots with 1, 2, and 3 mM salicylic acid increased essential oil percentage. They concluded that salicylic acid has an important role as a plant growth regulator and can improve essential oil yield and content in this plant. In another study, Abrunet al. (2016) found that applying 1 mM salicylic acid increased essential oil yield of summer savory, compared to control. Talaat and Laila (2010) investigated the effects of three amino acids including proline, ascorbic acid and thiamin on gladiolas, and found that applying 200 ppm proline had the highest effect on vegetative growth, flowering parameters, and plant chemical combination. Azarnivand et al. (2010) investigated the effect of applying spermidine on vegetative growth of chamomile plants and reported increased flower essences in the treated plants. Talaat et al. (2005) reported that spraying 50 ml salicylic acid increased essence content and yield in geranium.

Compared to control, the treatments with salicylic acid and amino acid proline significantly increased all the essential oil components of summer savory plants in this study, but resulted in significant reduction of α-pinene. Probably, there is a negative correlation between Carvacrol and α-pinene and they are considered as substrate and intermediate compounds in biosynthetic pathway. Amino acid proline and salicylic acid increased Carvacrol by decreasing α-pinene production. Terpenoids are the main constituents of essences and nitrogen is their substrate. Therefore, proline amino acid which improves nutrient (and also nitrogen) uptake and has an important role in synthesis of sugars and carbohydrates, significantly increases the essence yield as compared with control (Talaat and Balbaa, 2010; Singh, 2015). Ghassemi-Golezaniet al. (2018) showed that application of salicylic acid to *Carum copticum* L. increased the amounts of essential oil components such as thymol and γ-terpinene. El-Goharyet al. (2015) demonstrated that amino acid had positive effects on the physiologic indices and main components of savory essential oil including γ-terpinen, carvacrol and p-cymene.

Conclusion

Foliar application of different concentrations of salicylic acid and amino acid proline had significant effects on savory essential oil components and its morphological and physiological properties. Spraying proline significantly increased plant height, stem diameter, freshand dry weights of shoots, freshand dry weights of leaves, and number of flowers. Among the different levels of amino acid proline, 1.5 g/L resulted in the highest effect on most of the studied traits including plant height, stem diameter, freshand dry weights of leaves, and number of flowers. Also, among the different levels of salicylic acid, 1 mM led to the highest effect on most of the studied traits including number of side-stems, freshand dry weights of shoots, and essential oil percentage.The highest essential oil yield was obtained in the treatment with 3 g/L proline. The most typical components of plant essential oil were γ-terpinene and Carvacrol. There was a negative correlation between Carvacrol and α-pinene and they were considered as substrates and intermediate compounds in the biosynthetic pathway. Proline and salicylic acid decreased α-pinene production and therefore, increased Carvacrol levels. According to the findings, foliar application of proline speeded up the plant growth and photosynthesis due to rises in the uptake of nutrients (including nitrogen), synthesis of growth hormones, and its regulating effects. In addition, it was concluded that foliar application of salicylic acid, through increasing the degree of stomatal opening, improvedthe growth and photosynthesis rates, which eventually led to an increase in growth indices and overall performance of savory plant.

Acknowledgement

The authors would like to thank all who assisted in conducting this work. The present paper was retrieved from PhD thesis.This work is partially supported by Islamic Azad University of Saveh, Iran.

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