Morpho-physiological and biochemical properties of *Carum copticum* (L.): effects of salicylic acid

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Abstract

Salicylic acid (SA) is a phenolic compound and its foliar application shows regulatory effects on plants. In this research, Ajowan (*Carum copticum*) plants were treated with 0, 0.5, and 1 mM SA and proline, total phenol, chlorophylls, carotenoids, soluble sugar, N, P, and K contents were studied. Results showed that application of SA affected both quantitative and qualitative traits of Ajowan. The plants treated with SA (0.5 and 1 mM) showed higher plant height, number of umbels per plant, stem diameter, number of branch, number of seed per umbel, seed yield, a thousand kernel weight, and percentage and yield of essential oil compared to control plants. With supplementation of SA and increasing its concentration, total phenol, chlorophylls, carotenoids, and soluble sugar contents were also significantly enhanced. Results also indicated that application of SA significantly improved the amount of macro-element (N, P, and K) in Ajowan compared to untreated plants. In sum, application of SA as a foliar agent resulted in a remarkable change in morpho-physiological and biochemical traits of Ajowan plants.

Keywords: Ajowan; essential oil; foliar application; salicylic acid

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Introduction

Ajwain (*Carum copticum L.*) belongs to Apiaceae family that has been used as a rich source of bioactivity materials at the pharmaceutical level (Bairwa et al., 2012). Because of its essential oil, the aroma of ajwain has a highly positive impact on its medicinal and organoleptic properties. Some researcher have shown that essential oils extracted from ajwain plants has antioxidant, antibacterial, sporicidal, and antifungal activity (Moein et al., 2015). Furthermore, ajwain oil also can be used as an effective ingredient in prevention of male gestation (Paul and Kang, 2012). Vitali et al. (2016) showed that thymol is the most important active agent in ajwain oil; therefore, it has biological effects like antimicrobial and antioxidant activity. Moreover, positive effects of thymol as a preservative agent in foodstuff was also confirmed (Vitali et al., 2016). Flavor and fragrance industry worldwide is a billion dollar market growing at the rate of 18 billion per year (Bano et al., 2016) and

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essential oil has a special place in this worldwide business.

Salicylic acid, 2-hydroxybenzoic acid, is a natural signal molecule that shows defense mechanism in plants (Nazar et al. 2011). The reaction of plants to different stress factors can be regulated by SA, so in drought, chilling, heat, salt, ultraviolet, pathogens, and disease resistance conditions SA can improve plants tolerance to biotic and abiotic stress factors (Bideshki and Arvin, 2010). Plants' resistance to these stresses is increased by increasing protective compounds. SA accelerates production of protective compounds such as betaine, glycine, and praline in plant tissues (Zamaninejad et al., 2013).

Biochemical and physiological parameters improve under the influence of growth regulating agents, which in turn increase the quantity and quality of essential oils in the herbage industry (Bano et al., 2016). Several studies have demonstrated that salicylic acid plays a very valuable role as a growth regulator in important physiological processes such as photosynthesis, flowering, plant material absorption, growth regulation, and resistance mechanisms to environmental and biological stresses (Nivedithadevi et al., 2012).

Due to the induction of antioxidant responses protecting the crop against damage (Senaratna et al., 2000) and photosynthesis and nutrient content, the application of SA has increased (Khan et al., 2010). As suggested by Palma et al. (2013), SA and its related compounds have a significant role in the modulation of redox balance and elevated level of glutathione content.

In *A. thaliana*, application of exogenous SA acts as a signaling molecule. Therefore, SA generates and amplifies the tolerance of the plant in the oxidative stress condition. Metwally et al. (2003) in their study on the impact of SA on the stress adaptation in plants reported that plant species, concentration, method of usage, and treatment time affected adaptation to stress and curing to damage in plants. Gharib (2006) sprayed SA on sweet basil and found that the content of essential oil increased at 0.1 mM concentration. Jafari and Hadavi (2011) demonstrated that the optimum concentration of SA was between 0.1 mM and 2 mM. Since the application of SA is an efficient way for enhancing plant adaptation against different stresses, the present study aimed at identifying the effect of 0, 0.5, and 1 mM SA on quantitative and qualitative traits of ajwain (*Carum copticum*) in saline soil.

Materials and Methods

Soil characteristics

This study was carried out on a silt loam soil (Table 1) during summer 2016. Hydrometer method was used for determination of soil texture (sand, silt, and clay contents) (Bouyoucos, 1962). Moreover, pH meter, electrical conductivity (EC), and the wet oxidation method were employed to identify pH, EC, and organic matter (OM), respectively (Nelson and Sommers, 1996). The total nitrogen (N) was assayed using the method described by Bremner (1996). Moreover, available phosphorus (P) and potassium (K) were determined according to the procedure reported by Olsen (1954) and Helmke and Sparks (1996), respectively.

Experimental site, soil preparation, planting, and harvesting

All experiments were done in the Medicinal Plants Farm of Shahid Bakeri Higher Education Center of Miandoab (36.5°N latitude, 46.6°E longitudes, and an altitude of 1314 meters above the sea level). Seeds of ajowan (*Carum copticum*) were planted in the prepared field at a depth of about 10-mm with a spacing of 4 cm and a row spacing of 50 cm. Seeds were carefully irrigated after planting. After emergence of two

Table 1
Physico-chemical characteristics of the studied soil

Soil Properties	Value
Soil texture class	Silt/loam
Sand (%)	25
Silt (%)	49
Clay (%)	26
Organic matter, OM (%)	1.36
Total nitrogen, N (%)	0.13
PH of saturated paste	8.3
Electrical conductivity of saturated extract, EC_e (dSm^{-1})	2.1
Olsen (sodium-bicarbonate extractable) phosphorus (mg kg^{-1})	13.24

real leaves, SA (0.5 and 1 mM) was sprayed at five stages every 15 days. Distilled water was used for standard control samples. During the preparation of the field and the period of plant growth from planting to harvesting, no chemical fertilizers, herbicides, pesticides, and fungicides were used. The plants were harvested at the seed stage for relevant assays.

Plant height and diameter of stem

After harvest, stem diameters were measured by a caliper at three points: center and two ends of the plant and the average of the three points were reported. The plant heights were also measured by a tape measure. The number of seeds and umbrella inflorescence per plant, one-thousand grain weight of seeds, number of seeds per umbrella inflorescence, number of stems, and fresh and dry weights of plants after harvesting were determined.

Essential oil extraction

The essential oil of the air-shade dried ajwain samples were extracted using the hydro-distillation in a Clevenger method described by Tarakemeh et al. (2012). Briefly, the dry samples (50 g) were weighed, powdered, and subjected to Clevenger for 4 hours. The extracted essential oil was then dehydrated using anhydrous sodium sulfate. The collected samples were analyzed by gas chromatography mass spectrometry after being placed in hermetical glass vials at 4° C. The extracted essential oil was calculated by following equation (Tarakemeh et al., 2012):

Yield (%) = (mass content of essential oil / leaf dry weight (mass)) × 100

Proline analysis

Proline content of leaf tissues was determined using the method described by Bates et al. (1973). Briefly, the homogenized fresh tissue of plant in 10 ml of aqueous sulpho-acetic acid (3%) was weighed to 5 g. Filtration of the obtained suspension was dine via whatman filter paper Grade 1. Two ml of acid-ninhydrin and concentrated acetic acid were mixed with equal volume of the filtrated sample in a test tube. Four ml was added to test tube, and then the absorbance of the sample was measured at 520 nm through a spectrophotometer following shaking (UV Jenway 6300). The free proline content was measured using L-proline standard.

Chlorophylls and carotenoids

The extracted chlorophyll and carotenoid contents of the leaf tissues were determined using the method described by Arnon (1949). Before extraction, fresh leaf samples were rinsed in clean deionized water for the removal of surface contaminations. Photosynthetic pigment was extracted from the 4th fully expanded leaves from the top of the plant at the end of the experimental period. Then, 0.1 g of a fresh leaf was ground in 5 ml 80% (v/v) acetone in dark. Afterward, the filtrate slurry was centrifuged at 5,000 × g for 10 min. Then, the absorbance of the sample was determined at 645, 663, and 470 nm against acetone as blank with a spectrophotometer, for Chlorophyll a, Chlorophyll b, and carotenoids concentrations, respectively.

Total phenol content

Following the method presented by Gao et al. (2000), the total content of phenolic compound was measured using Folin–Ciocalteu (FC). Then, 0.1 gram of each samples were ground in 2ml methanol and centrifuged at 5000 g for 10 minutes at room temperature. Afterward, 50 µl of the resulting extract, 450 µl distilled water, and 2.5 ml 10% Folin–Ciocalteu reagent were mixed. The mixture was kept in a dark place for 6 minutes. Then, 2 ml of 7.5% (w/v) Na_2CO_3 was added. The solution was incubated at room temperature in the dark for 1.5 h and the absorbance was recorded at 765 nm with a spectrophotometer. Gallic acid was used as standard and the values were presented as milligram GAE (Gallic acid standard) equivalents per g of dry weight (mg GAE/g dw).

Soluble carbohydrate content

Soluble carbohydrates were determined based on phenol sulfuric acid method (Arnon, 1949). First, 100 mg of the weighed samples were put into a boiling tube containing 5 ml of 2.5 N-HCl. The tubes were placed in a water bath for 3 hours. Upon completion of hydroxylation, the samples were cooled to room temperature. Solid sodium carbonate was used for neutralization of samples. The ultimate content was increased to 100 ml by adding deionized water and centrifuged. In a series of test tubes, 0.2, 0.4, 0.6, 0.8, and 1 ml of the standard were applied. Then, 0.1 and 0.2 ml of the solutions were poured into separate tubes. Each tube volume was increased to 1 ml by adding distilled water. Then, 1 ml and 5 ml of phenol solution and sulfuric acid (96%) were added to each tube, respectively before they were completely shaken. After 10 minutes, the tubes were put in a water bath at a temperature of 27± 2° C for 20 minutes. The absorbance of the samples was recorded at 490 nm. Finally, the total carbohydrate content of the samples was measured via the standard chart. The blank sample was prepared with 1 ml of water instead of phenol solution.

Leaf N, P, and K contents

Nitrogen, phosphorous, and potassium contents in each treatment were measured via the methods described by Hashmi et al. (2012). First, the leaves from each treatment were well washed and dried and then completely powdered. Samples were digested to measure phosphorus and potassium contents. In short, 0.5 g of powdered dry samples and 10 ml of concentrated nitric acid 65% were mixed in digestive tubes. Sample tubes with two tubes as control (nitric acid only) were stored for 12 hours without any treatment. After 12 hours, the tubes were heated for 3 h at 60° C and then, the temperature was slowly augmented up to 110° C. The completion of digestion was determined using the indication of color clarity (yellow amber) of samples. Afterwards, the samples were immediately cooled to room temperature. The ultimate content was increased to 100 ml by adding distilled water. This solution was used to measure the amount of phosphorus and potassium. Measurement of phosphorus was done by vanadium-molybdenum colorimetric method. In the orthophosphate ions in the acidic medium with vanadium-molybdate form the yellow complex of vanadium molybdate, which shows the maximum absorption at 430 nm, flame emission was used to measure potassium. Potassium was first measured using flame photometry and then its calibration curve was drawn (Hashmi et al., 2012). The leaf content was determined by the Kjeldhal method (Khalid and Shedeed, 2015). One gram of powdered dry samples was used in a Pyrex digestion tube (30 ml of concentration). Then H_2SO_4 was added with caution to 10 g potassium sulfate and 14 mg copper sulfate. The resulting mixture was placed on sand and gently heated with a low flame to make it boil. Then, further heating was applied to turn it into a clear and colorless solution. After cooling and diluting the solution with distilled water, the contents were transferred into an 800 ml Kjeldhal flask. The digestion flask was washed and a piece of catalyst tablet, 100 ml of granulated zinc, and 40% caustic soda were added, respectively. Next, the flask was connected with a condenser to a splash-head distillation apparatus. Then, 25 ml of 0.1 N sulfuric acid was collected in the receiving flask on distillation system. Upon being removed and following the completion of reaction, the solution was titrated against 0.1 N caustic soda solution via methyl red indicator to measure nitrogen.

Statistical Analysis

The experiments were repeated at least three times and the results were expressed as the mean values plus standard deviations. Data analysis was conducted using ANOVA in SPSS software (v. 20.0). Duncan's Multiple Range Test was used to measure the significant differences between treatments ($P< 0.05$).

Results

The vegetative growth properties of ajwain are given in Table 2. Results showed that SA in all concentrations significantly improved vegetative properties compared to the control

Table 2

Comparison of means for the impact of SA on the quantitative and qualitative traits of ajwain

salicylic acid treatments	Plant height(cm)	Diameter of stem	Number of branch	1000-seed weight (g)	Seed yield (kg.ha^{-1})	Essential oil %	Essential oil yield (lit.ha^{-1})
Control	50.62±1.68c	3.00±0.92c	3.40±0.00c	1.51±0.02c	709.33±3.78c	2.23±0.04c	15.97±0.26c
0.5 Mm	58.96±1.20b	3.87±1.44b	4.26±0.30b	1.59±0.02b	727.82±4.14b	2.73±0.04b	19.98±0.51b
1mM	64.47±3.44a	4.52±0.29a	5.00±0.20a	1.69±0.02a	744.12±2.82a	3.34±0.07a	24.91±.54a

* Means in each row or column followed by the same lowercase or capital letters are not significantly different (P<0.05) by Duncan's Multiple Range Test.

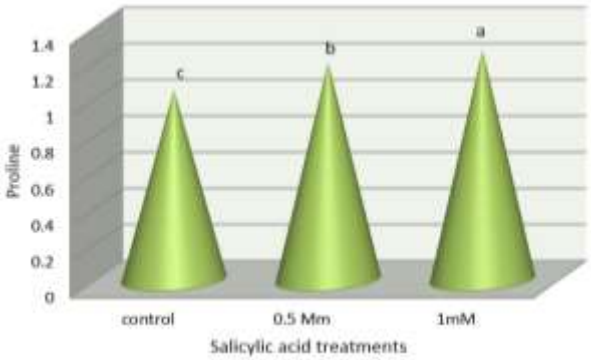

Fig. I. The effect of salicylic acid treatments on proline of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

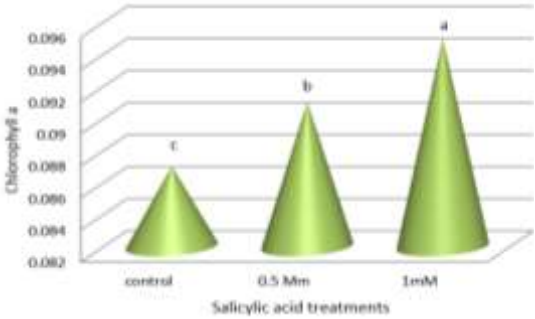

Fig. II. The effect of salicylic acid treatments on Chlorophyll a content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

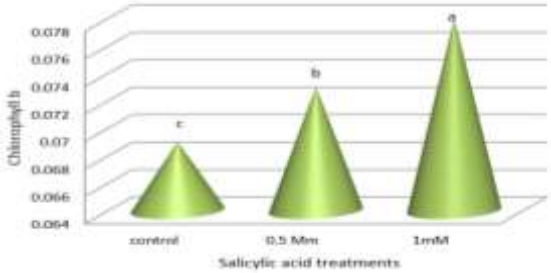

Fig. III. The effect of salicylic acid treatments on Chlorophyll b content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

(P<0.01). SA at 1 mM was more effective than 0.5 mM. The height of plants, number of branches, and diameter of stem were increased by 27.4%, 47.1%, and 50.6%, respectively. Table 2 also summarizes the effects of applying SA on 1000-seed weight of ajwain. Results indicated that 1000-Seed weight was strongly affected by SA. Moreover, the effect of different concentrations of SA on seed yield is presented in Table 2. The essential oil extracted from ajwain treated with SA was yellow (similar to the control). Findings of the essential oil in terms of dry matter (v/w) and oil yield (Lit/ha) are also presented in Table 2. The control sample had an essential oil content of 2.23%, whereas the essential oil contents of samples treated with SA at 0.5 and 1 mM were 2.73% and 3.34%, respectively.

The changes in proline contents of ajwain with foliar application of SA are presented in Fig. I. The findings indicated that the proline contents of the ajwain treated with 1 mM SA was 20.95 % more than the control plants.

Analysis of variance showed that SA application significantly changed chlorophylls (a and b) and carotenoids contents (Figs. II, III, IV).

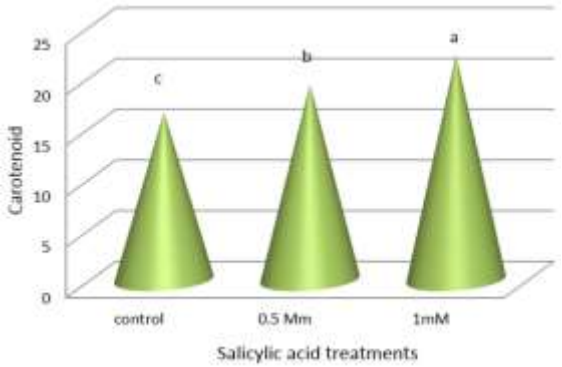

Fig. IV. The effect of salicylic acid treatments on carotenoid content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

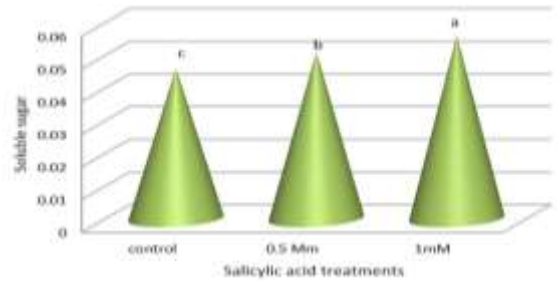

Fig. V. The effect of salicylic acid treatments on soluble sugar content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

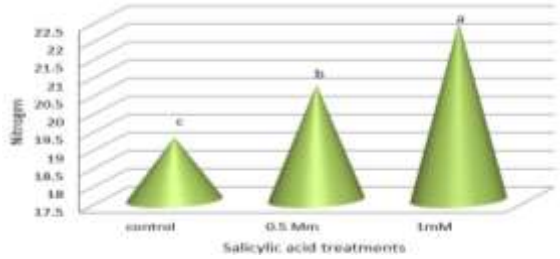

Fig. VI. The effect of salicylic acid treatments on nitrogen content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

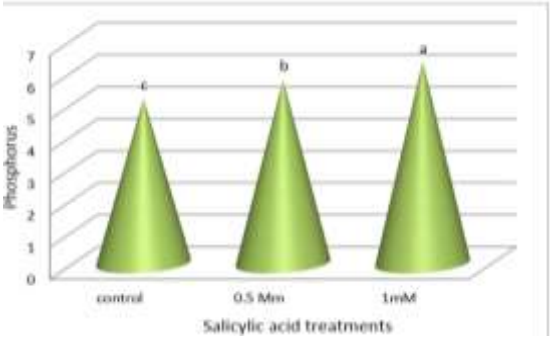

Fig. VII. The effect of salicylic acid treatments on phosphorus content of Ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of three replicates.

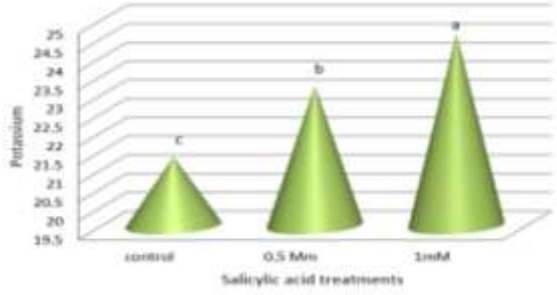

Fig. VIII. The effect of salicylic acid treatments on potassium content of ajwain; means followed by the same letter(s) are not significantly different at p<0.05 as determined by LSD test. The data shown are means of

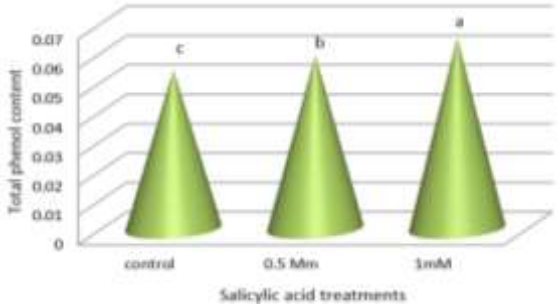

Fig. IX. The effect of salicylic acid treatments on total phenol content of ajwain; means followed by the same letter(s) are not significantly different at p < 0.05 as determined by LSD test. The data shown are means of three replicates.

The maximum content of photosynthetic pigments was observed in samples treated with SA (1 mM) while the minimum contents were observed in the control.

In addition, the correlations between total soluble sugars (TSS) of the samples are given in Fig. V. Results indicated that the foliar application of SA on ajwain significantly increased TSS content (P<0.05). Also, foliar application of SA had a positive impact on the nitrogen, phosphorous, and potassium contents of ajwain leaves (Fig. VI, VII, VIII). Finally, analysis of variance showed a significant difference in total phenol contents of SA treatments as compared with the control (Fig. IX).

Discussion

Application of SA had a significant impact on the regulation mechanisms of physiological and biochemical processes, so that it could increase the growth by regulation of cell division and changing proteins associated with the cell growth. Plant growth and development were reported to improve and SA eventually led the increased plant height (Idrees et al., 2010). Hashmi et al. (2012) also reported that an increase in vegetative growth of Fennel (*Foeniculum vulgare Mill*) with exogenous use of SA in different concentrations. Similarly, Mohammadzadeh et al. (2013) observed an enhancement in plant height, branch number, and stem diameter of four cultivars of basil (*Ocimum basilcum*) after SA treatments.

As compared to the control, 1 mM SA significantly enhanced 1000-seed weight (g) by 50.1% (P<0.05). This might be due to the improvement in the plant growth as a result of

application of SA. Similar results were reported for barely (*Hordeum vulgare*) (El-Tayeb, 2005).

Application of SA led to a significant increase in seed yield ($P<0.05$). Angourani et al. (2017) studied the effect of exogenous SA on various properties of pumpkin (*Cucurbita pepo L. var. Styriaca*) and found that SA treatment increased the seed yield about 21.1%. The significant enhancement of essential oil content was obtained through foliar spraying of 1 mM SA. Idrees et al. (2010) also presented similar results for thyme (*Thymus daenensis Celak*). They found that SA had an impact on the enhancement of growth cycle, nutrients uptake, leaf oil gland population, and monoterpenes biosynthesis and developed content of essential oil in the plants.

The impact of foliar application of SA on the oil yield of ajwain is summarized in Table 2. In comparison to control samples, oil yield was significantly increased by exogenous application of SA. The SA supplementation at 1 mM recorded the highest oil yield (24.91 Lit/ha). The lowest oil yield (15.97 l/ha) was achieved for untreated samples. The obtained results are comparable to the research reported by Zamaninejad et al. (2013) using the foliar application of SA on corn (*Zea mays* L.) that showed effective plant growth and production activity of SA on the plant.

Also, by increasing SA concentration, photosynthetic pigments were increased. This is mainly due to the enhancement of photosynthetic activity (Ramesh et al., 2002). By increasing SA concentration, total soluble sugars (TSS) were enhanced and thus the highest content of TSS was observed for 1 mM of SA. This is attributed to the increase in photosynthetic activity of the plant as a result of foliar application of SA (Simaei et al., 2011). The findings of the study are consistent with the results reported by Bybordi et al. (2018) for onion who concluded that SA significantly increased photosynthetic carbohydrate in leaves. Furthermore, the statistical analyses of the obtained data showed that the interaction between SA concentration and macro-element (N, P, and K) contents was significant. Supplementation of 1 mM SA increased N, P, and K contents by 16.4%, 22.7%, and 0.2%, respectively compared to untreated samples which has been mostly attributed to the enhancement of photosynthesis process (Hashmi et al., 2012). Similarly, increase in macro-elements by SA has also been found by Nazar et al. (2015) in mustard. Pirbalouti et al. (2013) showed that foliar application of SA reduced the negative effect of water deficit on thymol content in the essential oil of T. *daenensis*. The essential oils of T. daenensis exhibited antioxidant and antibacterial activities when plants were sprayed with 1.5 and 3.0 M SA, respectively (Pirbalouti et al., 2014). As they concluded, the use of salicylic acid in medicinal plants increases the phenolic compounds of their essential oils.

Conclusion

SA is a compound that has a significant role in plants. The findings showed that the growth and productivity of the ajwain were significantly affected by foliar application of SA. The supplementation of ajwain by different levels of SA brought about significant changes in its quantitative and qualitative traits. The obtained results revealed that the growth and yield of the plant were significantly increased by SA treatment. These results were due to the enhancement of photosynthetic activity by SA application so that the plant growth and other parameters under investigation improved. In conclusion, SA is an appropriate agent in acceleration of ajwain yield in all fields. The results of this study along with those reported by other researchers on the physiological role of salicylic acid showed that SA may regulate proliferation and cell division with other substances such as auxin (Nourafcan, 2014). Also this compound increases photosynthesis by increasing the activity of the RubisCO enzyme as well as the chlorophyll content, thereby increasing the dry weight of the plant (Hassanzadeh et al., 2016).

The phenolic compounds of salicylic acid facilitate the absorption of nutrients and have a positive role in photosynthetic activities and photosynthetic enzymes. This valuable substance causes the formation of cell wall pectin, the synthesis of malic acid, cell division, and the transfer of sugars and enzymes (Mashayekhi and Atashi, 2012).

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