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Assessment Effects of Different Level of Amino Acid and Seaweed Extract on Growth Traits and Essence Components of Sweet Scented Geranium (*Pelargonium graveolens* L.)

Shabnam Roshani¹, Hossein Ali Asadi-Gharneh^{*2}

1- MSc. Graduated, Department of Horticulture, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran.

2- Assistant Professor, Department of Horticulture, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran.

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ABSTRACT

BACKGROUND: Sustainable agriculture today plays an important role in the utilization of organic matter and biofertilizers to eliminate or reducing chemical fertilizer, promoting fertility and preserving biological activities, enhancing the health of agricultural products and reducing environmental damage.

OBJECTIVES: Current research was conducted to assessment physiological characteristics of sweet scented geranium affected different seaweed extract and amino acid.

METHODS: This study was carried out in greenhouses research of Islamic Azad University of Isfahan Branch (Khorasgan) via factorial experiment based on completely randomized design with three replications. The main factor included four level of seaweed extract (S_1 =nonuse of seaweed extract or control, S_2 =0.25, S_3 =0.5, S_4 =1 mg.l⁻¹) and second factor consisted four level of amino acid (A_1 = nonuse of amino acid or control, A_2 =1, A_3 = 1.5, A_4 =2 mg.l⁻¹).

RESULT: Mean comparison result showed the increase of shoot dry weight by use of (15.8 g) in 0.5 mg. Γ^1 seaweed extract (15.8 gr) and 2 mg. Γ^1 amino acid (16 g). Interaction effect of treatment had significant and additive effect on vegetative traits such as plant height. The highest leaf greenness index was observed by 0.5 mg. Γ^1 seaweed extract with 2 mg. Γ^1 amino acid. Aromatic essence of current study included linalool, menthol, betacitronellol, geraniol, cytonelin fumarate, beta-caryophylline, germacron, phenyl ethyl tiglate, geraniol tiglate was improved due to apply treatments. The highest beta-citronellol content was obtained of 1 mg. Γ^1 amino acid with nonuse of seaweed extract treatment.

CONCLUSION: Concentration of 2 mg. l^{-1} amino acid plus 1 and 1.5 mg. l^{-1} of seaweed extract achieved maximum quality and quantity of aromatic geranium.

KEYWORDS: Beta-citronellol, Carotenoid, Chlorophyll, Geraniol, Linalool.

1. BACKGROUND

The growing trend of diseases led to attention medicinal plants in community health and the use of bio-fertilizers due to the health of the crop and lower accumulation of chemicals in plant organs. Nutritional management also plays an important role in enhancing the production and quality of medicinal plants. The global approach to the production of medicinal plants has been directed towards improving the quantity, quality and health of the active ingredient. Therefore, it seems that the proper nutrition of these plants through the application of biological fertilizers is most in line with the production goals of the medicinal plants and can improve their quantitative and qualitative yield (Darzi et al., 2007). The essences have been considered because of their various antimicrobial activities including antifungal, antiviral, antibacterial, insecticidal and antioxidant properties. Pelargonium species is an essential plant belonging to the family Geraniaceae, which due to its medicinal benefits, is used in traditional medical systems so that out of 25 species, only four species, P. graveolens, P. odoratissium, P. capitatum and P. radens are of particular importance in the production of essences. Geranium essences one of the most expensive essence in the perfume, flavoring and cosmetics industries, so it is widely planted (Miller, 2002; Peterson et al., 2006; Rajasulochana et al., 2009; Sharopov et al., 2014). Essence content in aromatic plants is affected by various factors such as plant genetics, leaf life, nutrition and harvest time (Sangwan et al., 2001). Nutrition and fertilization

may be the most controllable factors that can influence the growth and composition of horticultural products (Kang and van Iersel, 2004). In modern management practices, the use of organic materials such as vermicompost and amino acids have improved soil fertility, increased microbial diversity, improved soil moisture and crop yield. Amino acid, as an organic matter, is effective in enhancing plant function by affecting the physiological activities of growth. In recent years, use of seaweed has been explored in modern agriculture (Erulan et al., 2009). Extracted bioactive substances from seaweed extract have been used in agricultural and horticultural crops around world and many of its beneficial effects have been reported, especially in enhancing quantitative and qualitative yield of various crops (Craigie, 2011). Unlike chemical fertilizers, extract obtained from seaweed prevents environmental degradation, is non-toxic and prevents contamination to humans and animals (Del Pozo et al., 2007).

2. OBJECTIVES

Current study was done to assessment physiological and biochemical traits of sweet scented geranium affected different seaweed extract and amino acid.

3. MATERIALS AND METHODS

3.1. Greenhouse and Treatments Information

This study was carried out in greenhouses research of Islamic Azad University of Isfahan Branch (Khorasgan) to assessment physiological characteristics of sweet scented geranium affected different seaweed extract and amino acid according factorial experiment based on completely randomized design with three replications. The first factor included the four level of seaweed extract (S₁=nonuse of seaweed extract or control, S₂=0.25, S₃=0.5, S₄=1 mg.l⁻¹) and the second factor consisted four level of amino acid (A₁= nonuse of amino acid or control, A₂=1, A₃= 1.5, A₄=2 mg.l⁻¹).

3.2. Greenhouse Management

In order to reduce the test error in each replicate, 2 pots (total of 96 pots) with volume of 4 liters were considered. In this experiment, used of folammina amino acid, made by Biolchim Italy, contains 19 amino acids (14% organic nitrogen, 14% water soluble organic nitrogen, 40% bio-based organic carbon, 2.85% C/N ratio, 68.8 organic mater, 6.5-7 pH, organic and pH value of about 7.5-5) were used. The seaweed used was algae type (containing 44.5%) organic matter, 1% alginic acid, 1 mg.1⁻¹ gibberellin acid, 19.3% water-soluble potassium oxide with pH between 8-8.8). Sweet Scented Geranium cuttings with 4 to 5 leaves, 12 to 10 cm long, were planted in 4-kg pots containing cocopit: Perlite in equal proportions. For irrigation and fertilization, 100 ml of nutrient solution was provided daily to the plants. To prevent salinity of the culture medium, water was replaced with nutrient solution at the end of each week. After establishment cuttings, treatments were spray foliar solution at 15 days intervals.

3.3. Measured Traits

The indices studied were vegetative, physiological and biochemical traits. Vegetative traits including plant height, number of leaves and branches, leaf area, shoot and root dry weight, leaf relative water content, also physiological and biochemical traits including chlorophyll a, b, leaf carotenoid content and aromatic essence compounds. The leaf relative water content was obtained by dividing the fresh and dry weight differentiation on its fresh weight (Mortazavi *et al.*, 2015).

Equ. 1. Leaf relative water content= FW-DW/FW × 100.

FW= Fresh weight, DW= Dry weight. Leaf area was measured by using the leaf area meter device (Model AM350). Leaf chlorophyll and carotenoid content was measured according Lichtenthaler and Wellburn (1987) method. Leaf chlorophyll was extracted with using 80% ethanol at 75 °C for 10 minutes. This procedure was repeated until complete extraction of the chlorophyll in the sample. Light absorption was measured by spectrophotometer at wavelengths of 470, 647, 664 and 700 nm. Finally, total chlorophyll and carotenoid were calculated by Equations (2) and (3).

Equ. 2. Chlorophyll_{a+b} =5.24 (A₆₆₄ - A₇₀₀) +22.24 (A₆₄₇ - A₇₀₀)

Equ. 3. Carotenoides= $(100(A_{470})- 2.86 (mg chl. A)-12.92 (mg chl. b))/245$

Leaf greenness was also evaluated by Spade. Assessment the composition of aromatic essence was measured by gas chromatography method (GC-Mass). For this purpose, essence extracted by clonger machine after preparation was injected into GC/MS to recognize type of their compounds. The Gas chromatograph type was Agilent 6890 with a 30 m column, 0.25 mm internal diameter and 0.25 µm thicknesses. The initial temperature of the oven was 50 °C and stopping at this temperature for 5 minutes, the thermal gradient of 3 °C per minute, the temperature rise to 240 °C at 15 rpm, and the temperature rise to 300 °C and it was 3 minutes stopping at this temperature. Injection chamber temperature was 290 °C and helium gas was used as carrier gas at a flow rate of 800 ml.min⁻¹. The mass spectrograph used was Agilent 5973 model with 70 eV voltage, EI ionization method and 220 °C ionization source temperature. The spectra were identified by their inhibition index method and compared with scientific literature using mass spectra of standard compounds.

3.4. Statistical Analysis

Analysis of variance and mean comparisons were done via SAS software (Ver.9) and Duncan multiple range test at 5% probability level.

4. RESULT

4.1. Shoot dry weight

According result of analysis of variance effect of seaweed extract and amino acid on shoot dry weight was significant at 5% probability level but interaction effect of treatments was not significant (Table 1). Mean comparison result of different level of seaweed extract indicated that maximum shoot dry weight (15 gr) was noted for 0.5 mg.l⁻¹ and minimum of that (11.5 gr) belonged to control treatment (Fig.1). As for Duncan classification made with respect to different level of amino acid maximum and minimum amount of shoot dry weight belonged to 2 mg.l⁻¹ (15.5 gr) and control treatment (12 gr), respectively (Fig.2).

4.2. Percentage of shoot dry matter

Result of analysis of variance showed effect of seaweed extract, amino acid and interaction effect of treatments on percentage of shoot dry matter was not significant (Table 1).

4.3. Root dry weight

According result of analysis of variance effect of seaweed extract, amino acid and interaction effect of treatments on root dry weight was significant at 1% probability level (Table 1). Evaluation mean comparison result of interaction effect of treatments indicated maximum root dry weight (3.43 gr) was for 0.5 mg.l⁻¹ seaweed extract and 2 mg.l⁻¹ amino acid and lowest one (1.1 gr) belonged to 0.25 mg.l⁻¹ seaweed extract and control treatments (Table 2).

4.4. Root dry matter percentage

According result of ANOVA effect of seaweed extract and interaction effect of treatments on root dry matter percentage was significant at 1% probability level but effect of amino acid was not significant (Table 1). Assessment mean comparison result of interaction effect of treatments indicated maximum root dry matter percentage (27.98%) belonged to 0.5 mg.l⁻¹ seaweed extract and 2 mg.l⁻¹ amino acid and minimum of that (9.85%) was for 0.25 mg.l⁻¹ seaweed extract and nonuse of amino acid treatments (Table 2).

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S.O.V	df	Shoot dry weight	Percentage of shoot dry matter	Root dry weight	Root dry matter percentage	Leaf dry weight
Seaweed Extract (SE)	3	35.96*	7.02 ^{ns}	3.58**	104.65**	0.25 ^{ns}
Amino Acid (AA)	3	31.84*	5.90 ^{ns}	1.12**	16.50 ^{ns}	0.15 ^{ns}
SE×AA	9	8.64 ^{ns}	3.41 ^{ns}	0.76**	68.92**	0.01 ^{ns}
Error	32	8.95	9.55	0.23	17.83	0.29

Table 1. ANOVA result of measured traits

^{ns}, * and ** are non-significant and significant at 5 and 1% probability levels, respectively.

	Continue Table 1.						
S.O.V	df	Leaf water content	Plant height	Number of leaves	Number of branch shoots	Leaf area	
Seaweed Ex- tract (SE)	3	8.18 ^{ns}	13.46**	67.20 ^{ns}	0.91 ^{ns}	2424.7 ^{ns}	
Amino Acid (AA)	3	14.36 ^{ns}	51.63**	38.65 ^{ns}	0.29 ^{ns}	8169.6 ^{ns}	
SE×AA	9	11.15 ^{ns}	7.06**	61.31 ^{ns}	0.35 ^{ns}	6537 ^{ns}	
Error	32	20.96	1.95	80.14	1.69	55229	

^{ns}, * and ** are non-significant and significant at 5 and 1% probability levels, respectively.

Continue Table 1.						
S.O.V	df	Chlorophyll a	Chlorophyll b	Carotenoid	Leaf green- ness index	
Seaweed Extract (SE)	3	0.003 ^{ns}	0.03 ^{ns}	0.02 ^{ns}	191.18**	
Amino Acid (AA)	3	0.02 ^{ns}	0.02 ^{ns}	0.04 ^{ns}	102.64**	
SE×AA	9	0.01 ^{ns}	0.01 ^{ns}	0.05 ^{ns}	10.25*	
Error	32	0.507	0.11	0.14	4.09	

^{ns}, * and ** are non-significant and significant at 5 and 1% probability levels, respectively.

4.5. Leaf dry weight

Result of analysis of variance revealed effect of seaweed extract, amino acid and interaction effect of treatments on leaf dry weight was not significant (Table 1).

4.6. Leaf water content

According result of analysis of variance effect of seaweed extract, amino acid and interaction effect of treatments on leaf water content was not significant (Table 1).

4.7. Plant height

According result of ANOVA effect of seaweed extract, amino acid and interaction effect of treatments on plant height was significant at 1% probability level (Table 1). Evaluation mean comparison result of interaction effect of treatments indicated maximum plant height (36.33 cm) was for 0.5 mg.l⁻¹ seaweed extract and 2 mg.l⁻¹ amino acid and lowest one (26.08 cm) belonged to 0.25 mg.l⁻¹ seaweed extract and nonuse of amino acid treatments (Table 2).

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Seaweed Extract (mg.l ⁻¹)	Amino Acid (mg.l ⁻¹)	Root dry weight (gr)	Root dry matter percentage	Plant height (cm)	Leaf greenness index
	0	1.26ef	16.74cde	27.33def	19.21f
0	1	1.34ef	17.42bcd	27.41def	19.27fg
0	1.5	1.60def	13.93de	28.50bcdef	19.66fg
	2	1.84cdef	18.18de	29.50bcd	20.66efg
	0	1.1f	9.85e	26.08f	18.47g
0.25	1	1.49def	14.35de	27.50cdef	22.06 defg
0.25	1.5	2.26cd	23.73abc	28.50bcdef	25.23bcd
	2	2.74abc	24.40ab	30.41b	28.74ab
0.5	0	2.18abc	20.14ab	26.92def	22.04 defg
	1	3.21ab	22.94abc	27.75bcdef	22.88 def
	1.5	1.94cdef	17.94bcd	30.41b	27.64bc
	2	3.43a	27.98a	36.33a	32 a
1	0	2.74abc	22.18abc	26.75ef	22.03 defg
	1	2.65abc	21.05abcd	28.58bcdef	23.43 de
1	1.5	2.37bcd	18.13bcd	28.83bcde	24.33cde
	2	2.28cd	22.10abc	30.33b	27.90 bc

Table 2. Mean comparison interaction effect of treatment on measured traits

*Similar letters in each column show non-significant difference at 5% probability level via Duncan test.

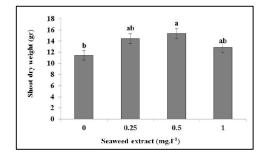


Fig.1. Effect of different level of seaweed extract on shoot dry weight by Duncan test at 5% probability level.

4.8. Number of leaves

Result of analysis of variance revealed effect of seaweed extract, amino acid and interaction effect of treatments on number of leaves was not significant (Table 1).

4.9. Number of branch shoots

According result of analysis of variance effect of seaweed extract, amino acid and interaction effect of treatments on number of branch shoots was not significant (Table 1).

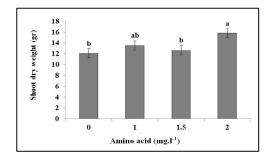


Fig.2. Effect of different level of amino acid on shoot dry weight by Duncan test at 5% probability level.

4.10. Leaf area

Result of analysis of variance revealed effect of seaweed extract, amino acid and interaction effect of treatments on leaf area was not significant (Table 1).

4.11. Chlorophyll a

According result of analysis of variance effect of seaweed extract, amino acid and interaction effect of treatments on chlorophyll a was not significant (Table 1).

4.12. Chlorophyll b

Result of analysis of variance revealed effect of seaweed extract, amino acid and interaction effect of treatments on chlorophyll b was not significant (Table 1).

4.13. Carotenoid

According result of ANOVA effect of seaweed extract, amino acid and interaction effect of treatments on carotenoid was not significant (Table 1).

4.14. Leaf green ness index

Result of ANOVA indicated effect of seaweed extract, amino acid and interaction effect of treatments on leaf green ness index was significant at 1% and 5% probability level, respectively (Table 1). Assessment mean comparison result of interaction effect of treatments indicated maximum leaf green ness index (32) was noted for 0.5 mg.l⁻¹ seaweed extract and 2 mg.l⁻¹ amino acid and lowest one (18.47) belonged to 0.25 mg.l⁻¹ seaweed extract and nonuse of amino acid treatments (Table 2).

4.15. Effect of seaweed extract and amino acid on mean amount of aromatic essence components

The major constituents of aromatic essence sweet scented geranium in this experiment were linalool, menthol, beta-citronellol, geraniol, cytonelin fumarate, beta-caryophylline, germacron, phenyl ethyl tiglate, geraniol tiglate concentration. The effect of different levels of seaweed extract and amino acid on the mean percentage of linalool showed the highest mean of linalool achieve in 1 mg.l⁻¹ application

of seaweed extract plus 2 mg.l⁻¹ amino acid. So, by application treatments, the concentration of linalool increased from 2.89% in control to 4.04% S₄A₄ (Fig. 3). According to Fig. 4, the effect of different levels of seaweed extract and amino acid on the percentage of menthol in aromatic essence showed a 2.23% increase in the amount of menthol per use of 2 mg.l⁻¹ amino acid and non-application of seaweed extract. The highest amount of the beta-citronellol (30.38%) was observed in the nonapplication of seaweed extract with 1 mg.1⁻¹ amino acid and the lowest one (24.94%) was observed in 0.5 mg.l⁻¹ seaweed extract with 1 mg.1⁻¹ amino acid (Fig. 5). According to Fig. 6, the effect of different levels of seaweed extract and amino acid on the percentage of geraniol showed a 4.73% increase in the amount of menthol per use of 0.25 mg.1⁻¹ seaweed extract and nonuse of amino acid. The highest amount of cytonelin fumarate (13.8%) was observed in the 0.25 mg.l⁻¹ of seaweed extract with 1 mg.l⁻¹ amino acid and the lowest one (10.31%) was observed in 0.25 mg.l⁻¹ seaweed extract with nonuse of amino acid (Fig. 7). According to Fig. 8, the effect of different levels of seaweed extract and amino acid on the percentage of beta-caryophylline showed a 1.15% increase in the amount of betacaryophylline per use of 0.25 mg.l⁻¹ seaweed extract and nonuse of amino acid. The highest amount of germacron (5.74%) was observed in the 1 mg.l⁻¹ of seaweed extract with 1 mg.1⁻¹ amino acid and the lowest one (0.47%) was observed in 0.5 mg.l⁻¹ seaweed extract with 1.5 mg.¹ amino acid (Fig. 9).

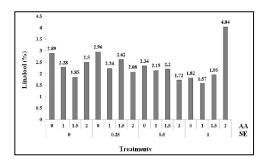


Fig. 3. Effect different level of seaweed extract (SE) and amino acid (AA) on linal-ool concentration.

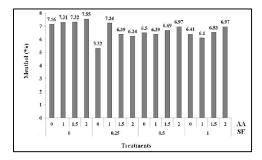


Fig. 4. Effect different level of seaweed extract and amino acid on menthol concentration.

According to Fig. 10, the highest amount of phenyl ethyl tiglate (3.18%) was observed in control treatment. Also the highest amount of geraniol tiglate (3.58%) was found in 1 mg.l⁻¹ seaweed extract with 1 mg.l⁻¹ amino acid (Fig. 11).

5. DISCUSSION

Based on the above results, it can be concluded that seaweed extract and amino acid treatments led to increase shoot and root dry weight due to growth stimuli. Also the reason for increased shoot growth, plant height and leaf area can be attributed to further root development.

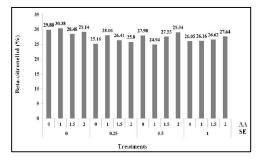


Fig. 5. Effect different level of seaweed extract and amino acid on beta-citronellol concentration.

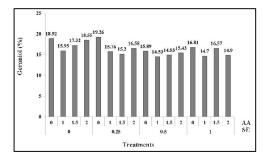


Fig. 6. Effect different level of seaweed extract and amino acid on geraniol concentration.

Abdel-Mawgoud et al. (2010) by evaluation the effect of different levels of seaweed extract (at concentrations of 0, 1, 2 and 3 g.1⁻¹) in three watermelon cultivars reported that seaweed extract due to compounds such as betaine (with cytokinin-like activity) led to increased growth of shoot and root. Also Mahfouz and Sharaf-Eldin (2007) reported a positive effect of organic matter on significant increase in fennel plant height, which was similar with the results of this study. Increased vegetative growth of plants such as beans and tomatoes due to use seaweed extract confirmed by some researchers (Sivasankari et al., 2006; Zodape et al., 2011).

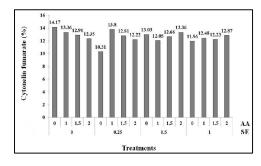


Fig. 7. Effect different level of seaweed extract and amino acid on cytonelin fumarate concentration.

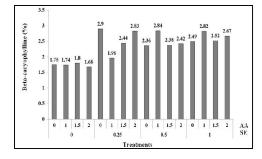


Fig. 8. Effect different level of seaweed extract and amino acid on beta-caryophylline concentration.

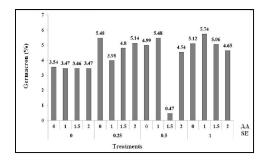


Fig. 9. Effect different level of seaweed extract and amino acid on germacron concentration.

Some researcher such as Khan *et al.* (2009) also reported increasing the concentration of seaweed extract led to increase the root growth and uptake nutrition elements so the plant could improving the plant growth.

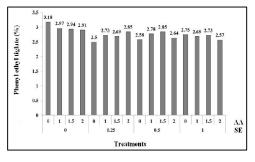


Fig. 10. Effect different level of seaweed extract and amino acid on phenyl ethyl tiglate concentration.

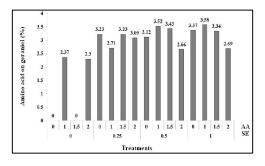


Fig. 11. Effect different level of seaweed extract and amino acid on geraniol tiglate concentration

Alam et al. (2013) reported that foliar application of seaweed extract has a significant effect on root development and growth of various plant species including strawberry, winter rapeseed and coastal pine. It seems Positive effects of using seaweed extract with stimulating properties on geranium led to increase shoot/root ratio, maximum leaf number, increased water uptake efficiency and nutrient uptake by roots. Sridhar and Rengasamy (2011) reported the effect of seaweed extract (at concentrations of 0, 0.25, 0.5, 1.5, 2%) on fresh weight, dry weight, root length, stem length, leaf area, chlorophyll content, protein and carbohydrate yield were positive and significant. The seaweed extract has

increasing effect on the shoots dry weight, while the effect of amino acids on the growth of the shoots is ineffective, which can be explained by the role of seaweed hormones in plants. Some researchers reported that the use of biofertilizers had a significant effect on the number of branches, plant height, and height of the first branch, which is similar to the results of this study (Choulwar et al., 2005; Çamaş et al., 2012). Abdel-Mawgoud et al. (2010) reported that seaweed extract increased all vegetative parameters such as plant length, number of leaves and number of shoots and had a significant difference at 5% level with control. Some researchers have reported an increase in the number of leaves or branches due to the growth-promoting substances such as hormones (cytokinin, indole-butyric acid, indole acetic acid), vitamins, amino acids, elements such as iron, copper, zinc, molybdenum, manganese and Nickel in seaweed extract (Erulan et al., 2009, Kotnala et al., 2009, Thambiraj et al., 2012). Amino acids improve the quantity and quality of crops by increasing the percentage and amount of chlorophyll in plants, promoting photosynthetic activity of plants and increasing C/N ratio (Geshnezjany, 2011). Seaweed extract has a significant effect on the physiological and chemical indices of plants due to the high amount of photosynthetic pigments induced by growth stimuli such as cytokinin, auxin and betaine (Schwab and Raab, 2004; Abdel-Mawgoud et al., 2010). It has been found that betaine may act as a nitrogen source at low concentrations of N (Khan et al., 2009). There are also reports of the treatment

of geranium extracts with seaweed extract due to increased photosynthetic pigment accumulation that can maintain the yield of green leaves and thus improve plant yield. Treating geranium cutting with seaweed extract is due to increased photosynthetic pigment accumulation which can maintain the yield of green leaves and thus improve plant yield. Seaweed extract also reduces aging by increasing chlorophyll content and preventing its degradation (Erulan et al., 2009). Aromatic essence of sweet scented geranium in current study included linalool, menthol, betacitronellol, geraniol, cytonelin fumarate, beta-caryophylline, germacron, phenyl ethyl tiglate, geraniol tiglate. Mentioned essences are valuable when they contain higher amounts of citronellol, and any environmental factor that enhances vegetative growth of the plant has an adverse effect on aromatic essence content (Mithila et al., 2001). In this study, the highest beta-citronellol content was obtained of 1 mg.1⁻¹ amino acid with nonuse of seaweed extract treatment. Sani (2010) reported that vermicompost and amino acid compounds can affect the growth and vield of some medicinal plants such as basil, garlic, fennel, ash and chamomile and have positive effects on yield and flower essence of medicinal plants. The results of current study showed that the treated compounds had no effect on the concentration of cytonelin fumarate and phenyl ethyl tiglate, but the treatment 1 mg.l⁻¹ amino acid and seaweed extract increased 3.58% geraniol tiglate concentration.

Spraying on amino acids requires further studies, and some sources have not been identified. According to the results, it can be concluded that different nutritional treatments can affect essence yield.

6. CONCLUSION

The results of current experiment indicated the superiority of the seaweed extract and amino acid over the control and the positive reaction of the main components of the aromatic geranium essence to these treatments. Concentration of 2 mg.l⁻¹ amino acid plus 1 and 1.5 mg.l⁻¹ of seaweed extract achieved the maximum quality and quantity of aromatic geranium. Mentioned result was observed in studied traits such as shoot dry weight, root dry weight, relative leaf water content and plant height. Also, the combination of seaweed and amino acid extracts increased the percentage of linalool, germacron and phenyl ethyl tiglate in comparison with the separate treatments.

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FOOTNOTES

AUTHORS' CONTRIBUTION: All authors are equally involved.

CONFLICT OF INTEREST: Authors declared no conflict of interest.

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