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Application of Plant Biostimulants to Improve the Biological Responses and essential oil production of marjoram (*Majorana hortensis*, Moench) plants

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ABSTRACT

A pot experiment was conducted at the nursery of the Ornamental Horticulture Dept., Fac. of Agric., Cairo Univ., during the two successive seasons of 2014/2015 and 2015/2016, to study the effect of some bio-stimulants (humic acid at 2.5 and 5 m/L, chitosan at 2.5 and 5 g/L and brassinolide at 25 and 50 ppm) on the vegetative growth, oil yield and oil constituents of marjoram plant (*Majorana hortensis*, Moench.). The data showed that plant height, branches number, fresh and dry weights of herb as well as essential oil yield per plant in second cut were significantly higher than the first one. The first cut gave insignificantly higher percentage of essential oil than the second one. Pigments content in second cut were significantly higher than that first one. In most cases, application of the three bio-stimulators at the two concentrations significantly increased the growth attributes of marjoram plants including plant height, number of branches, fresh and dry weights of herb oil percentage and oil yield compared to control plants. Among the two different concentrations of bio-stimulators, the lowest concentration of chitosan, humic acids and brassinolide gave the highest values of plant height, number of branches, fresh and dry weights of herb, chlorophyll-a and carotenoids content than those recorded with the highest one in most cases, while the highest concentration of the three bio-stimulators was more effective for increasing oil percentage, oil yield/plant and chlorophyll-b than the lowest concentration. GLC analysis indicated that essential oil extracted from plants treated with chitosan at the two levels, humic acid at high level (5 ml/l) and brassinolide at 25 ppm were found to be rich in cis-sabinene hydrate, whereas terpinen-4-ol was found to be rich in oil of plants treated with humic acid at 2.5 ml/l or brassinolide at 50 ppm. Also, both levels of chitosan increased the linalool percentage in the oil Sabinene and γ-terpineol, decreased with all treatments as compared with the control. The highest content of carbohydrates was recorded in the plants treated with the high concentration of bio-stimulators, and chitosan was the most effective one. From the obtained results it can be concluded that humic acid, chitosan and brassinolide are recommended for improving plant growth, oil yield and main components of *Majorana hortensis*, plants and the highest concentration of these three bio-stimulators was more effective for increasing oil percentage and oil yield/plant.

Key words: *Majorana hortensis*, Bio-stimulator, Brassinolide, Chitosan and Humic acid.

Introduction

Marjoram (*Majorana hortensis*, Moench), sweet marjoram, belonging to family Lamiaceae, it is a perennial aromatic herb native to North Africa, southwest Asia and Southern Europe. The plant reaches a height of 40-50cm and has small, gray-green, ovate leaves and pink- purple flowers. Marjoram plant is the most favor economic agriculture export crop. Marjoram is cultivated in Egypt, it is adapted to well-drained, fertile loam soils. The aerial parts of the plant are used for extraction of essential oil, which has a lot of uses in flavor (Tiziana and Dorman, 1998). Harvesting is done at full bloom and can be done two or three times per year. The essential oil contains terpinen-4-ol, α-terpinene, α-terpineol, γ -terpinene, cis-sabinene hydrate, linalool and several other compounds (Abd El –Wahab, 2013).The major compounds of the volatile oil of marjoram leaves obtained by (GC–MS) are terpinen-4-ol, γ-terpinene, trans-sabinene hydrate and linalool, trans-sabinene hydrate acetate and thujanol (Soliman *et al* 2009). The oil is used in perfumery and pharmaceutical industry in bruises, sprain, stiffs, paralytic limbs, acute diarrhea and as a spice, processed vegetables, condiments(

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Burdock, 1995).The marjoram herb contains terpenoids, phenols and flavonoids as major constituents (Janicsak *et al*. 1999), and others like steroids, fatty acids and vitamins (Baâtour *et al*. 2011).

In the last few years there has been a growing interest in the use of natural bio-stimulators for enhancing the growth of medicinal and aromatic crops. The activity of bio-stimulators to promote and regulate plant growth and development is often attributed to their ability to directly or indirectly provide mineral nutrients to plants that improves plant productivity (Yakhin, *et al*., 2016). The application of bio-stimulators increases plant growth directly via phytohormone action and also influences the plant's ability to control its own hormone biosynthesis and homeostasis. The use of natural bio-stimulators has extensively used in modern agricultural production, especially medicinal and aromatic herbs, to intensify the quality,quantity of crop yield and to ensure safe both for human beings and for the environment (Dhargalkar and Pereira, 2005). Humic acid, chitosan and brassinosteroids are among of such bio- stimulators.

Humic acid (HA) has a positive effects and several benefits on plant growth, production and absorption of nutrients by plants [Chen and Avoid, 1990 and Khalil and Yousef, 2014]. The treatment with humic materials increased root growth in a manner similar to auxins (Donnell 1973). It has been found that oregano plants sprayed with potassium-humate at 1% stimulated plant growth, yield and nutrient uptake (Said-Al Ahl, *et al*. 2009). On *Thymus vulgaris* treated with HA at 3 g/L resulted increase in essential oil yield and composition, antioxidant activity, total phenolic and flavonoid contents (Juárez *et al*. (2011). HA increased carbohydrate content in treated turnip (*Brassica rapa*) plants (El-Sherbeny *et al*. (2012). Treating *Calendula officinalis* with HA at 2 g/L increased the number of leaves, dry weight, and plant height (Mohammadipour *et al*. 2012). Application of HA at 1.5 l/ha, gave the highest number of branches and camphor content in *Rosmarinus officinalis* (Nia, *et al*. (2014), while HA application at 2- 5 g/l, increased height, number of branches, shoot dry weight and essential oil and enhanced chlorophylls content during both cuts of *Mentha piperita var. citrata* and basil plants (Hendawy *et al*. 2015, Jamali *et al*.. 2015 and Vafaei *et al*. 2015). Also, other investigators found that spraying dill plants by HA gave the highest values of plant height, number of branches and umbles and oil yield (Said-Al Ahl, *et al*. 2016) and its spray on *Guizotia abyssinica* resulted in a significant increase in plant growth and oil and protein contents (Tadayyon *et al*. 2017).

Chitosan is a natural biopolymer has been proven to stimulate and increase nutrient uptake, chlorophyll content, photosynthetic and chloroplast enlargement, oil yield and its composition can lead to the synthesis of secondary metabolites, such as polyphenols, lignin and flavonoids (Hadwiger 2013 ; Malekpoor, *et al*., 2016 and Salachna, *et al*., 2017). The stimulating effect of chitosan on plant growth may be attributed to an increase in the availability and uptake of water and essential nutrients through adjusting cell osmotic pressure and increasing enzyme activities (Guan *et al*. 2009). Chitosan increased key enzymes activities of nitrogen metabolism (nitrate reductase, glutamine synthetase and protease) and improved the transportation of nitrogen (Gornik *et al*. 2008). On *Ruta graveolens* it has been stated that, chitosan significantly increased growth rate and induced a significant increase in the oil concentrations (Orlita, *et al*. 2008). Application of chitosan at 200 and 500 ppm promoted plant height growth of *Majorana vulgare* ssp. hirtum, whereas 50 and 200 ppm regulated the content of polyphenols (Yin *et al* 2012). On fennel plants, El-Bassiony *et al*., (2014) indicated that foliar spray of chitosan gave the highest leaves number, dry weight of leaves and total yield. On Snapdragon plants application of chitosan significantly increased total carbohydrates and N, P and K % in plant organs (El- Attar, 2017). Massoud *et al*., (2016) on *Coriandrum sativum* showed that chitosan significantly affected growth characters, fruit yield and essential oil productivity.

Brassinosteroids (brassinolide, BRs, steroidal compounds) are among the natural bio-stimulators and they have great potential and distinct physiological effects on plant growth and development, used as yield stimulators in agriculture. They occur in free form and conjugated to sugars and fatty acids and consider as highly promising, environment-friendly natural substances (Krishna, 2003 and Bhardwaj, *et al*., 2006). Several workers discussed the physiological effects of BRs on plants and revealed a wide plant physiological changes (Sakurai and Fujioka, 1993; Fujioka and Sakurai, 1997; Kang and Guo, 2010, and Bera *et al.* 2014). On medicinal and aromatic plants, previous worker found that, BRs increased fresh weight of leaves and menthol production in *Mentha arvensis* (Maia, *et al*., 2004). Application of spermidine and stigmasterol at100 ppm increased the plant growth and essential oil content of chamomile plant (Abd El-Wahed and Gamal El-Din, 2004). Application of stigmasterol at 50 or 100 mg/L on *Tagetes erecta*, increased growth, essential oil, total nitrogen, carbohydrates and piperitone contents (Balbaa *et al.*2008). On *Pelargonium graveolens* application of stigmasterol at 50, 100 and 150 mg/L increased fresh and dry weights, plant height ,crude protein, essential oil% and the content of citronellol and linalool (Ayad *et al.*(2009). On *Satureja khuzestanica* it has been found that, foliar application of brassinolide increased herbage yield, rate of photosynthesis, chlorophyll, carbohydrate fractions (reducing and non-reducing sugars and starch) and the content of essential oils (Eskandari and Eskandari (2013). Recent research on *Lathyrus odoratus*, (Metwally, et al. 2016), found that progesterone applied at at 10, 20 and 30 ppm showed promotive effect on number of branches, dry weight/plant, oil % and significantly increased beta-ocimene. Also, on *Mentha arvensis* application of BRs significantly improved the yield of menthol, menthone and menthyl acetate (Naeem, *et al*. 2012 and Naeem, *et al*. 2017).

The aim of this study was to investigate the effect of some bio-stimulators, including; humic acid, chitosan and brassinolide on *Majorana hortensis*, Moench plants, to improve plant growth, oil yield and main components and providing growers with useful information about the response of majorana plants to these bio-stimulators.

Material and Methods

Apot experiment was carried out at the Experimental Nursery of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt, during the two successive seasons of 2014/2015 and 2015/2016. This study was designed to investigate the effect of foliar application of three commercial bio-stimulators (humic acid, chitosan and brassinolide) on vegetative growth and essential oil characters of marjoram (*Majorana hortensis*, Moench.) plants.

Experimental procedure:

Seeds of marjoram plants (kindly obtained from the Dept. of Medicinal and Aromatic Plants, Ministry of Agric., Egypt.) were sown on 15th October, on the foam trays containing a mixture of sand: clay (1:1 by v/v). After 45 days, uniform seedlings (10-12 cm length) were individually transplanted in clay pots (30 cm diameter) filled with 7-8 kg of a clayloam soil (Table, 1). The plants were grown under field conditions and irrigated every 8-10 days.

Table 1: The physical and chemical characteristics of the soil mixture

<table>
<tr><td colspan="8">Physical characteristics 2014/2015/2015/2016</td></tr>
<tr><td>Clay(%)</td><td>Silt(%)</td><td>Fine sand (%)</td><td>Coarse sand (%)</td><td rowspan="3">Texture Clay loam</td><td>pH</td><td>EC dS/m</td><td>Organic matter</td></tr>
<tr><td>38.30</td><td>30.40</td><td>22.00</td><td>9.30</td><td>8.1</td><td>1.6</td><td>1.56</td></tr>
<tr><td>36.50</td><td>25.70</td><td>31.60</td><td>6.20</td><td>7.6</td><td>1.4</td><td>1.88</td></tr>
<tr><td colspan="8">Chemical characteristics</td></tr>
<tr><td colspan="4">Soluble Cations (meq/l)</td><td colspan="4">Soluble Anions (meq/l)</td></tr>
<tr><td>Ca</td><td>Mg</td><td>K</td><td>Na</td><td>Cl-1</td><td>SO4-</td><td>Available N (ppm)</td><td>Available P (ppm)</td></tr>
<tr><td>7.7</td><td>2.6</td><td>1.27</td><td>2.8</td><td>4.6</td><td>2.32</td><td>37</td><td>22</td></tr>
<tr><td>7.2</td><td>2.9</td><td>1.33</td><td>1.2</td><td>5.5</td><td>2.44</td><td>45</td><td>29</td></tr>
</table>

Experimental treatment:

After 15 days from transplanting from 15th December till 15th August (in both seasons), the plants were sprayed every 3weeks with the following bio-stimulators: humic acid, chitosan and, brassinolide at two rates , using bio-film at 1 ml /L as a wetting agent.The plants were sprayed by a hand sprayer until run off point. The following concentrations of bio-stimulators treatments were: Control, humic acid at 2.5 and 5.0 ml/L, chitosan at 2.5 and 5.0 g /L and brassinolide at 25 and 50 mg/ L. All bio-stimulators products were obtained from the Union for Agriculture Development Co., UAP, and Egypt. All the plants were monthly received a dose of 2.5 g /pot of N-P-K fertilizer "Life Green; 20-20-20).

Experimental design:

The layout of the experiment was a complete randomized block design, with 3 replicate (each replicate contain 4 plants).So, the study included7 treatments (including the control) with 84 plants (12 plants /treatment).

Harvesting:

The plants were harvested twice times in both seasons by cutting the aerial parts of each plant at 8-10 cm above the soil surface. The two cuts were done on the 15th of April and 30 th August, in both seasons.

Data recorded:

Vegetative growth:

On both seasons, randomly samples were taken from each treatment at each cut and the following vegetative growth parameters were recorded**:** Plant height (cm), number of branches/plant as well as fresh and dry weights of herb/plant (g/ plant).

Extraction and GLC analysis of essential oil:

Oil percentage (%) was determined in the dried herb as indicated by Egyptian Pharmacopeia (1984) by distilling the herb for 2.5 - 3.0 hours in order to extract the essential oil (50 g of dried herb). The oil content was calculated by multiplying oil percentage by weight of dry herb (ml/plant). Gas Liquid Chromatography analysis (GLC) was used to analyze the essential oil constituents. The extracted essential oil was dehydrated over anhydrous sodium sulphate and stored at freezer till used for analysis.The dehydrated oil was separately subjected to GLC analysis at the Central Laboratory of Cairo University. The retention time (RT) of the components was used to identify the constituents of the essential oil, using authentic samples under the same conditions, according to Guenther (1961) and British Pharm. (1980).

Determination of chemical constituents of the herb:

Chlorophylls (a and b) and carotenoids contents (mg/g F.W) were estimated on fresh matter basis according to Saric *et al*., (1967).The total carbohydrates content (% of dry matter) were determined in dried herb sample using the procedure of Dubois, *et al.* (1956).

Statistical analysis:

Data recorded on vegetative growth and chemical compositions were statistically analyzed. An analysis of variance (ANOVA) was carried out, and the means of the recorded data were compared using the "least significant difference (L.S.D.)" test at the 5% level, as described by Steel and Torrie (1980).

Results and discussion

Vegetative growth characteristics.

Plant height (cm).

The response of *Majorana hortensis*, Moench plants to bio-stimulators (Table2) showed that, in both seasons the data recorded on plant height of second cut (27.39 and 29.47 cm, in the first and second seasons, respectively) were significantly higher than those of the first cut (24.04 and 25.40 cm in both seasons, respectively). The data also indicated that, in both seasons supplying plants with humic acid, chitosan and brassinolide at the low concentration resulted in significantly taller plants compared to the control. The interaction effect showed that in the first and second cuts, in both seasons, supplying the plants with HA, chitosan and brassinolide each at the low concentrations, in most cases, gave taller plants than that obtained from plants supplied with the highest concentrations with only one exception recorded with application of brassinolide In the first cut which showed increase in plant height with raising the concentration from 25 to 50 ppm.

Number of branches/plants

Data in Table (2) show the number of branches/plants in response to the different treatments of bio-stimulators. In both seasons, the second cut of plants significantly formed more of branches than the first cut. The data indicated that all bio-stimulators, in the both seasons, increased the formation of branches and such increases were significant in most cases compared with the control. In first and second seasons, the application of humic acids, chitosan and brassinolide at the low concentrations gave more number of branches (11.91. 13.27, 11.56 .11.55, 11.86 and 12.05 branches/ plant, respectively) than the high level. The interaction effects between cuts and bio-stimulators showed that the treatment of humic acid at the low concentration, in both seasons (15.30 and 17.21 branches /plant) was the most effective on increasing the branches formation per plant followed by the low level of both chitosan and brassinolide. Khazaie, *et al*. (2011) on *Hyssopus officinalis* and Hendawy, *et al*.(2015) on *Mentha piperita* var. *citrata* plants observed that, humic acid had a significant effect on growth characters during both cuts.

Table 2. Effect of bio-stimulators on plant height and number of branches/plant of *Majorana hortensis* during the seasons of 2014/2015 and 2015/ 2016.

Bio-stimulators	First Season (2014/2015)			Second Season (2015/2016)		
	1stCut	2ndCut	Mean (B)	1stCut	2ndCut	Mean (B)
Plant height (cm)						
Control	20.93	22.60	21.76	24.23	24.10	24.16
Humic acid at 2.5 ml/l	24.10	31.67	27.88	26.17	34.27	30.21
Humic acid at 5.0 ml/l	23.43	25.20	24.32	25.56	26.33	25.95
Chitosan at 2.5 g/l	25.80	31.53	28.67	24.93	33.66	29.30
Chitosan at 5.0 g/l	22.73	25.13	23.93	24.33	26.33	25.16
Brassinolide at 25ppm	25.46	31.10	28.28	25.33	34.03	29.68
Brassinolide at 50 ppm	26.80	24.50	25.15	27.23	27.90	27.56
Mean (A)	24.04	27.39	-----	25.40	29.47	-----
L.S.D 5 %	A=3.00	B=3.02	AB=4.28	A=2.98	B=2.84	AB=4.03
Number of branches/ plant						
Control	6.20	7.90	7.05	8.00	10.03	9.02
Humic acid at 2.5 ml/l	8.53	15.30	11.91	9.33	17.21	13.27
Humic acid at 5.0 ml/l	7.66	10.10	8.88	7.77	12.36	10.06
Chitosan at 2.5 g/l	9.03	14.10	11.56	7.56	15.54	11.55
Chitosan at 5.0 g/l	9.30	10.63	9.96	8.13	12.80	10.47
Brassinolide at 25ppm	9.93	13.80	11.86	8.00	16.11	12.05
Brassinolide at 50 ppm	8.23	12.26	10.25	8.23	14.54	11.39
Mean (A)	8.41	12.01	----	8.15	14.09	----
L.S.D 5 %	A= 0.87	B=1.75	AB=2.47	A= 1.68	B=1.76	AB=2.48

Fresh and dry weights of herb(g) /plant

The averages of fresh and dry weights of herb per plant, of the two cuts of *Majorana hortensis* in response to bio-stimulators are shown in Table (3).The data showed that, in both seasons, there were significant differences between the first and second cuts for fresh weight of herb/plant, giving 32.87, 55.50, 37.48 and 60.73 g/plant, respectively. The same trend was recorded for the dry weight of herb, producing 18.63, 27.41, 22.81and 32.57 g/g plant, respectively. The data also indicated that, regardless the date of cuts, treating the plants with chitosan at the low contention and brassinolide at the high level were the most effective bio-stimulators treatments for increasing the fresh weight of herb, in both seasons, they resulted

Table 3: Effect of bio-stimulators on fresh and dry weights of herbs (g/plant) of *Majorana hortensis* during the seasons of 2014/2015 and 2015/ 2016.

Bio-stimulators	First Season (2014/2015)			Second Season (2015/2016)		
	1stCut	2ndCut	Mean (B)	1stCut	2ndCut	Mean (B)
Fresh weight of herb g /plant						
Control	23.29	29.44	26.37	25.96	36.22	31.09
Humic acid at 2.5 ml/l	28.13	64.12	46.13	29.40	65.51	47.46
Humic acid at 5.0 ml/l	35.38	53.98	44.68	35.20	63.37	49.29
Chitosan at 2.5 g/l	33.19	66.19	49.69	36.62	75.57	56.10
Chitosan at 5.0 g/l	35.52	54.34	44.93	38.28	57.12	47.70
Brassinolide at 25ppm	38.01	57.78	47.90	47.46	60.27	53.87
Brassinolide at 50 ppm	36.56	62.48	49.52	49.46	67.04	58.25
Mean (A)	32.87	55.50	----	37.48	60.73	----
L.S.D 5 %	A=1.16	B=5.72	AB=8.09	A=2.98	B=3.80	AB=5.38
Dry weight of herb g/plant						
Control	13.92	16.05	14.99	17.70	22.05	19.88
Humic acid at 2.5 ml/l	16.22	31.79	24.02	19.56	32.22	25.89
Humic acid at 5.0 ml/l	19.82	26.09	23.00	18.87	31.85	25.36
Chitosan at 2.5 g/l	19.17	32.55	25.86	24.79	41.94	33.37
Chitosan at 5.0 g/l	19.91	25.68	22.80	26.98	30.66	28.82
Brassinolide at 25ppm	22.41	29.93	26.17	24.39	32.40	28.40
Brassinolide at 50 ppm	18.93	29.78	24.36	27.40	36.86	32.13
Mean (A)	18.63	27.41	----	22.81	32.57	----
L.S.D 5 %	A=1.85	B=1.37	AB=1.94	A=3.05	B=1.78	AB=2.52

49.69, 49.52, 56.10 and 58.25 g/ plant, in the first and second seasons, respectively and humic acid had no clear trend. Regarding the effective bio-stimulators on dry weight of herb, the data indicated that treating the plants with the low contention of brassinolide in the first season and chitosan in the second one gave the heaviest dry herb, regardless the cut date. Concerning the interaction effect of bio-stimulators and date of cuts on fresh and dry weights of herb, the data generally cleared that, chitosan with the low concentration , in both seasons, increased fresh and dry weights of herbs giving the heaviest values (66.19, 75.57, 32.55 and 41.94 g /plant, respectively).

The obtained results clearly revealed that the second cut gave higher growth attributes than the first one (plant height, number of branches, fresh and dry weights of herb). The application of the humic acid, chitosan and brassinolide greatly increased the growth attributes of marjoram plants. Also, the above mentioned results revealed that treating the plants with the low concentrations of tested bio-stimulators (humic acid,chitosan and brassinolide) was the most effective for increasing the studied growth parameter. In this regard, Nardi *et al*. (2002) stated that humic acids may primarily increase growth by increasing cell elongation or cell membrane permeability, therefore increased water uptake by increased plant roots. Also, Chen and Avaid (1990) indicated that humic substances have a very pronounced influence on the growth of roots and growth of plant foliage. On the other hand, Orlita, *et al*. (2008) on *Ruta graveolens,* Yin, *et al*. (2012*)* on *Majorana vulgare*, El-Bassiony *et al.*(2014) on fennel and Mehregan *et al*.(2017) on *Stevia rebaudiana* they indicated that foliar spray of chitosan increased plant height, number of leaves and branches as well as fresh dry weights of stems, leaves and shoots.

The promotive effect of chitosan on growth parameter may be due to its effect on increasing nutrient uptake and availability, chlorophyll content and photosynthetic rate, through adjusting cell osmotic pressure and increasing enzyme activities (Hadwiger, 2013, Malekpoor, *et al*., 2016 and Salachna, *et al*. (2017). Additionally, Gornik *et al*.(2008) reported that chitosan increased enzymes activities of nitrogen metabolism (nitrate reductase, glutamine synthetase and protease) and improved the transportation of nitrogen. Our results concerning the effect of brassinolide on vegetative growth of marjoram plant are in a good agreement with the results obtained by Maia *et al*. 2004 on *Mentha. arvensis,* Abd El-Wahed and Gamal El-Din (2004) on chamomile*,* Balbaa *et al*. (2008) on *Tagetes* *erecta* ,Ayad *et al.* (2009) on *Pelargonium graveolens*, Eskandari, and Eskandari (2013) on *Satureja khuzestanica,* Metwally *et al.*(2016) on *Lathyrus odoratus* and Naeem, *et al.(*2017) on *Mentha arvensis* L. they found that the treatments of brassinolide substances increased fresh and dry weight of leaves, plant height and promoted number of branches. The promotive effect of brassinolide on growth paramter may be due to enhance the cells growth ,differentiation,enlargement and division, change membrane potentials and the metabolism of nucleic acids and proteins.(Clouse and Sasse,1998, Khripach, *et al.,*2002, Müssig, 2005 and Prins, *et al.,*2010).

Essential oil production.

Essential oil percentage (%)

Data presented in Table (4) showed that in both seasons, oil percentage in the first cut was insignificantly higher than the second one. The data also showed that oil percentage of *Majorana hortensis* plants was greatly affected by using of biostimulators treatments which significantly increased the values obtained in treated plants in most cases, comparing with the control. Application of brassinolide at 50 ppm was the most effective treatments, which gave the highest values (1.257and 1.077 % in the first and second seasons, respectively) followed by using humic acid at the high dose 5ml /L treatments (1.077 and 0.933%, in both seasons, respectively).

Table 4: Effect of bio-stimulators on essential oil percentage (% dried herb) and oil yield /plant of *Majorana hortensis* during the seasons of 2014/2015 and 2015/ 2016.

Bio-stimulators	First Season (2014/2015)			Second Season (2015/2016)		
	1stCut	2ndCut	Mean (B)	1stCut	2ndCut	Mean (B)
Oil % (dry herb)						
Control	0.586	0.694	0.640	0.506	0.726	0.616
Humic acid at 2.5 ml/l	0.726	0.684	0.710	0.600	0.666	0.633
Humic acid at 5.0 ml/l	1.160	0.984	1.077	1.040	0.826	0.933
Chitosan at 2.5 g/l	0.820	0.840	0.830	0.866	0.686	0.776
Chitosan at 5.0 g/l	0.880	0.894	0.887	0.814	0.746	0.780
Brassinolide at 25ppm	0.880	0.924	0.907	0.914	0.720	0.817
Brassinolide at 50 ppm	1.280	1.234	1.257	1.114	1.040	1.077
Mean (A)	0.91	0.89	----	0.84	0.77	----
L.S.D 5 %	A=0.073	B=0.146	AB=0.206	A=0.084	B=0.119	AB=0.188
Oil yield /plant (ml)						
Control	0.082	0.111	0.097	0.090	0.160	0.125
Humic acid at 2.5 ml/l	0.118	0.221	0.170	0.117	0.215	0.166
Humic acid at 5.0 ml/l	0.230	0.259	0.245	0.196	0.263	0.230
Chitosan at 2.5 g/l	0.157	0.273	0.215	0.215	0.288	0.252
Chitosan at 5.0 g/l	0.175	0.230	0.203	0.220	0.229	0.225
Brassinolide at 25ppm	0.197	0.280	0.239	0.223	0.233	0.228
Brassinolide at 50 ppm	0.242	0.367	0.305	0.305	0.383	0.344
Mean (A)	0.171	0.248	----	0.195	0.252	----
L.S.D 5 %	A=0.046	B=0.065	AB=0.092	A=0.050	B=0.053	AB=0.075

The application of chitosan significantly increased the essential oil percentage as compared with the control, the high dose was more effective than the low one. The interaction effect showed that in both seasons, supplying the plants with brassinolide at 50 ppm followed by humic acid at 5 ml/l .gave the highest values of oil percentage in the first cut of both seasons (1.280 and 1.114, 1.160, and 1.040 %, respectively). Generally, it can be mentioned that, the first cut gave higher percentage of essential oil than the second one. Spraying plants with the high level of bio-stimulators (humic acid, chitosan and brassinolide) greatly increasing the percentage of essential oil of marjoram plants than the lower one and control and the treatment with brassinolide at high level 50 ppm produced the highest percentage of essential oil (% dry weight) in the two cuts of both seasons. The results of increasing essential oil percentage due to brassinolide treatments are in agreement with finding of Youssef and Talaat (1998) on lavender, Abd El-Wahed and Gamal El-Din (2004) on chamomile plants, Balbaa *et al*. (2008) on

Tagetes erecta, Ayad *et al*.(2009) on *Pelargonium graveolens*, Eskandari and Eskandari (2013) on *Satureja khuzestanica*, Metwally, *et al*. (2016) on *Lathyrus odoratus*, whereas increasing essential oil percentage due to humic acid applications are in harmony with Hendawy *et al*. (2015) on *Mentha piperita var. citrata* and Jamali *et al*.(2015) on basil plants and Tadayyon (2017) on *Guizotia abyssinica*. Regarding the effect of chitosan, Orlita, *et al*. (2008) on *Ruta graveolens* and Massoud, *et al*., (2016) on *Coriandrum sativum* reported increases in essential oil productivity by chitosan treatments.

Essential oil yield (ml/plant).

Data presented in Table (4) indicated that the essential oil yield per plant of *Majorana hortensis* plants in the second cut of both seasons was significantly higher than that of the first one. The application of bio-stimulators treatments significantly affected the essential oil yield per plant, in both seasons, comparing with control. In both seasons, spraying the plants with brassinolide at 50 ppm was the most effective treatment, giving values of 0.305 and 0.344 ml/plant, in the first and second seasons, respectively. A pronounced increase in the essential oil yield per plant was recorded with the treatment of humic acid at5 ml/l in the first season (0.245 ml/plant) and low level of chitosan 2.5 g/l (0.252 ml/plant)in the second season Regarding the interaction, the obtained results revealed that spraying plants with brassinolide at high level (50 ppm) produced the highest values of oil yield /plant in the two cuts of both seasons , recording the following values 0.242 ,0.367, 0.305 and 0.383 ml/plant, respectively. Treating plants with humic acid at 5 ml/l, in the first cut of the first season, had a pronounced effect on increasing the oil yield /plant (0.230 ml), whereas in the second cut, the low level of both chitosan and brassinolide had a marked effect on increasing the oil yield /plant. In the second season, treating plants with humic acid at 5 ml/l and chitosan at 2.5 g/l markedly increased the oil yield /plant (0.263 and 0.288 ml/plant, respectively). Increases in oil yield as a result of humic acid treatments are in accordance with finding of Said-Al Ahl, *et al*. (2016) on dill plants. The increases in essential oil yield /plant by bio- stimulators treatments can be owing to the increase of oil % of dried herb and the improvement of herb yield (Mohammadipour *et al*.,2012).

Essential oil composition

The chromatograms (Fig.1-7) of *Majorana hortensis* oil, obtained from Gas Liquid Chromatography analysis (GLC), in the second cut of the second season, revealed that the identified main components are: terpinen-4-ol, cis-sabinene hydrate, sabinene, γ-terpineol, linalool, limonene, linalylacetate, γ -terpinene,and cis-sabinene hydrate. The data indicated that plants treated with chitosan at the two levels, humic acid at high level (5 ml/l) as well as brassinolide at 25 ppm were found to be rich in cis-sabinene hydrate, whereas terpinen-4-ol was found to be rich in essential oil of plants treated with humic acid at 2.5 ml/l or brassinolide at 50 ppm. Also, both levels of chitosan increased the linalool percentage in the oil, as compared with the other treatments and the control. Sabinene and γ-terpineol, decreased with all bio-stimulators treatments as compared with the control. Novak *et al*. (2002) reported that the formation of cis-sabinene hydrate and acetate seems to be a result of a specific enzymatic conversion in marjoram plants. Eskandari and Eskandari (2013) on *Satureja khuzestanica* found that the application of brassinolide caused a significant increase in the essential oil content and a slight increase in the content of carvacrol and para-cymene and a decrease in γ-terpinene, β-bisabolene and myrcene contents.

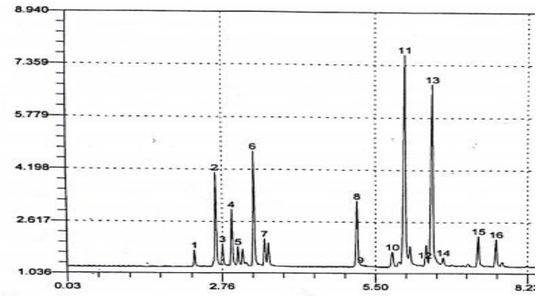

Fig. 1: Chromatogram of essential oil extracted from 2.5 ml/l humic acid

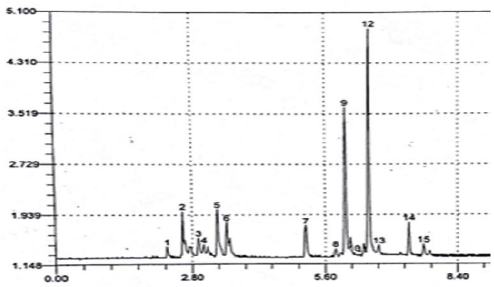

Fig. 2: Chromatogram of essential oil extracted from 5 ml/l humic acid

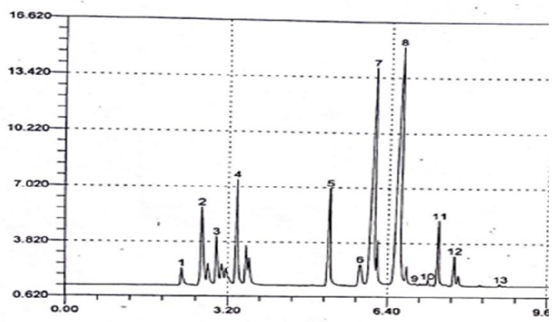

Fig. 3: Chromatogram of essential oil extracted from 2.5 g /l chitosan

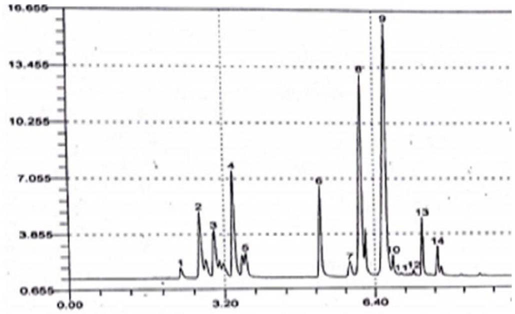

Fig. 4: Chromatogram of essential oil extracted from 5 g /l chitosan acid /

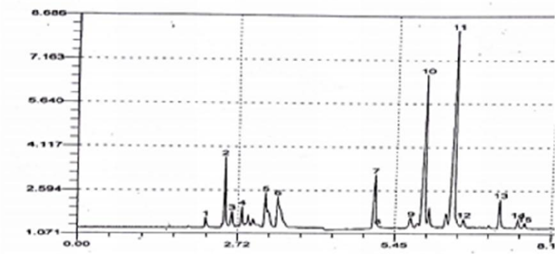

Fig. 5: Chromatogram of essential oil extracted from 25 ppm/l brassinolide

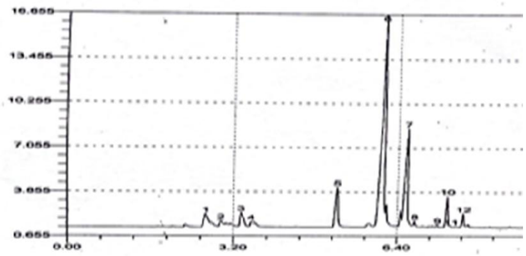

Fig. 6: Chromatogram of essential oil extracted from 50 ppm /l brassinolide

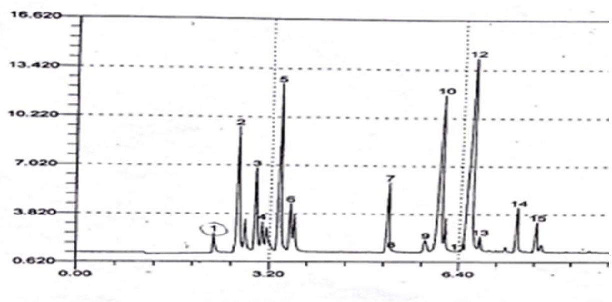

Fig. 7: Chromatogram of essential oil extracted from untreated plants (control)

Chromatograms (Figs. 1-7): GLC analysis of *Majorana hortensis* oil obtained from plants treated with different bio-stimulators, in the second cut of the second season, 2015/2016.

1-Phyllandrane, 2- Sabinene, 3- Pinene, 4- γ-Ter Pinene, 5- Limonene, 6- Linolool, 7- γ-Terpineol,

8- Terpinen-4-ol, 9- Cis- Sabinene, 10- Linalyl acetate 11- Thuyan-4-ol, 12- B-caryophyllene

Pigments content

As shown in Table (5) in both seasons, the plants of the second cut contained insignificantly higher chlorophyll-a,-b and carotenoids than the first one in most cases. Treating the plants with bio-stimulators generally increased the synthesis and accumulation of pigments content in herb of marjoram plants compared with the control. Application of the three tested bio-stimulators at the lowest concentration was most effective for increasing the content of chlorophyll a in the first season and carotenoids in both seasons in most cases. The high content of chlorophyll-b was recorded in the plants treated with the highest concentration of any of bio-stimulators, compared to the control. The treatments of chitosan and brassinolide were the most effective in increasing the content of chlorophylls during the two seasons. There are several reports of the increasing chlorophyll levels in plants due to brassinolide application such as Kandil, *et al*. (2007) on *Rosa hybrid*, Shahid *et al.* (2011) on *Pisum sativum*, Hu *et al*. (2013) on *Leymus chinensis,* Shalaby *et al***.** (2014) on *Hordeum vulgare*, Eskandari and Eskandari (2013) on *Satureja khuzestanica* and Swamy *et al* .(2014) on *Trigonella foneugraecum*, while increases chlorophyll levels as a result of chitosan application are in agreement with findings of Malekpoor *et al*. (2016) on *Ocimum basilicum*. Salachna *et al*.(2017) on *Verbena bonariensis*.

Table 5: Effect of bio-stimulators on chlorophyll-a ,b and carotenoids contents of *Majorana hortensis* during the seasons of 2014/2015 and 2015/ 2016.

Bio-stimulators	First Season (2014/2015)			Second Season (2015/2016)		
	1stCut	2ndCut	Mean (B)	1stCut	2ndCut	Mean (B)
Chlorophyll-a (mg/g F.W.)						
Control	1.21	1.32	1.26	1.18	1.32	1.25
Humic acid at 2.5 ml/l	1.19	1.36	1.28	1.26	1.44	1.35
Humic acid at 5.0 ml/l	1.21	1.27	1.24	1.35	1.35	1.36
Chitosan at 2.5 g/l	1.28	1.32	1.30	1.39	1.38	1.39
Chitosan at 5.0 g/l	1.22	1.30	1.26	1.47	1.44	1.46
Brassinolide at 25ppm	1.35	1.29	1.32	1.51	1.37	1.44
Brassinolide at 50 ppm	1.29	1.34	1.31	1.31	1.39	1.35
Mean (A)	1.25	1.31	----	1.35	1.38	----
L.S.D 5 %	A=0.09	B=0.13	AB=0.19	A=0.10	B=0.13	AB=0.18
Chlorophyll-b (mg/g F.W.)						
Control	0.28	0.78	0.53	0.42	0.76	0.59
Humic acid at 2.5 ml/l	0.68	0.67	0.68	0.53	0.66	0.59
Humic acid at 5.0 ml/l	0.70	0.83	0.77	0.53	0.67	0.60
Chitosan at 2.5 g/l	0.62	0.72	0.67	0.67	0.53	0.59
Chitosan at 5.0 g/l	0.67	0.82	0.75	0.69	0.60	0.65
Brassinolide at 25ppm	0.57	0.47	0.52	0.57	0.56	0.56
Brassinolide at 50 ppm	0.83	0.55	0.69	0.80	0.54	0.67
Mean (A)	0.62	0.69	----	0.60	0.62	----
L.S.D 5 %	A=0.12	B=0.15	AB=0.21	A=0.18	B=0.13	AB=0.18
Carotenoids (mg/g F.W.)						
Control	1.18	1.40	1.29	1.15	1.43	1.29
Humic acid at 2.5 ml/l	1.60	1.52	1.56	1.31	1.42	1.36
Humic acid at 5.0 ml/l	1.43	1.42	1.43	1.37	1.44	1.41
Chitosan at 2.5 g/l	1.49	1.54	1.52	1.63	1.38	1.51
Chitosan at 5.0 g/l	1.35	1.39	1.36	1.37	1.31	1.34
Brassinolide at 25ppm	1.50	1.35	1.43	1.44	1.44	1.44
Brassinolide at 50 ppm	1.38	1.47	1.42	1.35	1.40	1.38
Mean (A)	1.42	1.44	----	1.37	1.40	----
L.S.D 5 %	A=0.28	B=0.17	AB=0.22	A=0.27	B=0.24	AB=0.34

Total carbohydrates

The effects of humic acid, chitosan and brassinolide on total carbohydrates content in dried majorana herb (%) in the second cut of the first and second seasons, are shown in Fig.(8). The data revealed that in both seasons, the highest content of carbohydrates was recorded in the plants treated with the high concentration of bio-stimulators, and chitosan was the most effective one in this respect, compared to the control. The higher chlorophyll levels might have contributed to the increased levels of carbohydrates. This result was in accordance with Eskandari and Eskandari (2013) on *Satureja khuzestanica*, who found that foliar application of brassinolide increased rate of photosynthesis and carbohydrate fractions and they also indicated that higher levels of carbohydrates and their possible diversion to secondary metabolism might contribute to elevate levels of the essential oil in the plant.

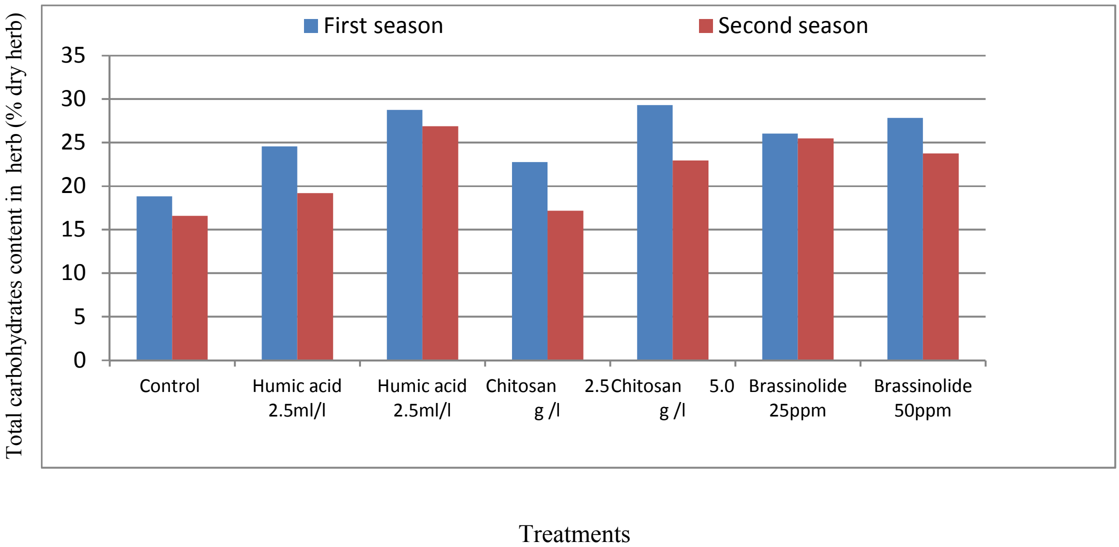

Fig. 8: Effect of humic acid, chitosan and brassinolide on total carbohydrates content in herb (% dry herb) in the second cut of the first and second season

Conclusion:

from the obtained results it can be concluded that humic acid, chitosan and brassinolide are recommended for improving plant growth, oil yield and main components of *Majorana hortensis*, plants and the highest concentration of these three bio-stimulators was more effective for increasing oil percentage and oil yield/plant.

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