Influence of Drought Stress, Biofertilizers and Zeolite on Morphological Traits and Essential Oil Constituents in *Dracocephalum moldavica* L.

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Abstract

Background: **Essential oil content and composition in medicinal and aromatic plants is highly affected by both biotic and abiotic factors.**

Objective: **To investigate the effects of drought stress, zeolite and bio-fertilizers on morphological characteristics and essential oil constituents in *Dracocephalum moldavica* L.**

Methods: **This experiment was conducted in the form of split factorial based on randomized complete block design with three replications in two years (2014 and 2015). The factorial experimental treatment was three levels of soil moisture (90%, 60% and 30%FC) applied to main plot, while application of zeolite (0 and 2 g per kg of soil) and bio-fertilizers (control, nitroxin, phosphate barvar-2 and nitroxin×phosphate barvar-2) were applied to sub plots.**

Results: **Application of zeolite had the highest effect on plant height, leaf area, dry matter and flowering top branches yield (FTBY) in 90%FC and essential oil percentage and yield in 60%FC. Similarly, combination of nitroxine+phosphate barvar-2 caused maximum value of plant height, leaf area, dry matter and FTBY in 90% FC and essential oil percentage and yield in 60%FC. The GC-MS analysis of the essential oil identified 14 compounds including ester and non ester combinations. The main compounds of ester combinations were geranyl acetate and neryl acetate and the main compounds of non ester combinations were neral, geraniol and geranial. The maximum percentage of ester and non ester combinations produced in 30%FC and 60-90%FC, respectively.**

Conclusion: **Application of zeolite accompanied by combination of nitroxine+phosphate barvar-2 produced the highest value of examined traits in *D. moldavica* L.**

Keywords: ***Dracocephalum moldavica*, Biofertilizer, Geranial, Geraniol, Neral, Water deficit stress**

Introduction

Dragonhead (*Dracocephalum moldavica* L.) is an annual and aromaticherbaceous, belonging to the Lamiaceae family [1]. The origin of dragonhead is reported from Himalaya and Siberia. This plant is acclimatization to temperature zones of Europe and Asia, naturally [2] and found in the northwest of Iran, Azerbaijan, Tabriz, Urmia, Yazd, Mazandran and Alborz mountain ranges [3]. It is widely used in pharmaceutics, traditional medicine, cosmetics, food industry and perfumery [4].

Drought is one of the most important environment factor that influence on production of crop plants in arid and semi-arid regions [5]. Drought stress causes different physiological effects on plant growth. So, irrigation treatments should be used effectively to increases the sustainability of production in agriculture systems [6]. There are many ways for drought tolerance that is used by plants such as rapid growth in different stage of plant, adaptation with tropical regions, relative resistance against dryness, high content of protein in leaves and high leaf area index (LAI), also high potential for enough production and high water use efficiency are suitable mechanism for drought tolerance [6]. Medicinal and aromatic plants have received much attention in several fields as agro aliment, perfume, pharmaceutical industries and natural cosmetic products [7]. Secondary metabolites in the medicinal and aromatic plants are conventionally affected by both gene types, and environmental factors [8]. It means growth parameter, essential oil yield and constituents of oil is impressed by biotic and abiotic environmental factors [9, 10].

Researchers and farmers use some methods to reduce injurious effects of water deficiency; zeolite application is one of the possible approaches to reduce effect of water deficit on plant production. Zeolite consists, alkali and alkaline materials and crystalline alumino silicate that have internal surface area for keeping water in drought durations. More than forty types of zeolite have been previously reported [11], these minerals have many properties which are interest for agricultural purpose; high caution exchange capacity, high water holding capacity and high adsorption capacity [12]. It has been confirmed that the suitability of using natural zeolite in agriculture had a positive role in plant nutrition and microbial community stability, as evidenced subject experimentation crops [13]. All these unique properties of zeolite materials promise to contribute significantly too many years of agricultural technology.

Recently, unconventional efforts are used to minimize the amounts of chemical fertilizers which applied to medicinal and aromatic plants in order to reduce production cost and environmental pollution without reduction of yield. Therefore, the trend now is using the bio and organic fertilizers. Bio-fertilizers are reasonably safer to the environment than chemical fertilizers and play an important role in decreasing the use of chemical fertilizers. Bio-fertilizers containing beneficial bacteria and fungi improve soil chemical and biological characteristics, phosphate solutions and agricultural production [14]. Bio-fertilizers have improved quantity and quality features of some plants [14-16]. Bio-fertilizers comprised of nitrogen fixers, phosphate dissolvers and available potassium. It has been acknowleged that inoculation of soil with bacterial mixtures caused a more balance nutrition for plants and improvement in root uptake of nitrogen and phosphorus in a main mechanism of interaction between phosphate solubilizing and bacteria nitrogen fixing [17]. Ratti et al. [15] investigated effect of some varieties of phosphate solubilizing bacteria on the yield of lemon grass and concluded that the plant height and biomass increased compared to the control condition. It has been reported that the use of phosphate solubilizing bacteria significantly increased the height of tea plant [18]. Gupta et al. [19] found that inoculation of mint root by mycorrhiza fungi has considerably increased the height and yield of plant. In another research, Kapoor et al. [20] showed that fennel root symbiosis with two species of VAM fungi significantly increased the flowering, 1000 seeds weight, dry matter and seed yield. They reported that the inoculation of root with a kind of phosphate solubilizing bacteria enhanced the shoot dry matter.

The present investigation was done in order to evaluate the effect of different level of water deficit, zeolite application and different types of bio-fertilizers on some quantity and quality features of dragonhead (*Dracocephalum moldavica* L.).

Materials and Methods

Site description and experimental set up

Two-year field experiment was conducted at the experimental farm of the Seed and Plant Improvement Institute in Karaj (latitude 35°48´N, longitude 51°26´E, altitude 1321 m above mean sea level) with relatively temperate and arid climate, and mean annual precipitation and temperature of 250 mm and 14.2 °C, respectively. The experiment was performed in the form of split factorial based on randomized complete block design with three replications during 2014 and 2015. The factorial experimental treatment was three levels of soil moisture (90%, 60% and 30% FC) applied to main plot, while application of zeolite (0 and 2 g per kg of soil) and bio-fertilizers (nitroxin, phosphate barvar-2 and nitroxine×phosphate barvar-2) were applied to subplots. The soil-water potential based on depletion of the available soil water was determined by a soil moisture release curve. Nitroxine (free-living nitrogen fixing bacteria) is a traditional name of biological nitrogen. Also, phosphate barvar-2 (phosphate solubilizing bacteria) was contained pseudomonas and bacillus (each mm contains 10^8 microorganisms). Land was deeply ploughed in the last autumn after being fallowed, and then was ploughed and hit again in the early spring andfinally was leveled by a trowel. Blocks were 4 m away from each other and plots were 1.5 m away from each other. The soil physiochemical characteristics of experimental farm is given in Table 1.

Seeds were sown at the depth of 0.5-1 cm, and thinning operations were performed at the end of the second week so that distance among plantson the lines was 12.50 cm and plant spacing among lines were 40 cm. No pest and disease species were observed in the farm and also no pesticides and fertilizers were used. After leaving out 1 m from the beginning

Table 1- The soil physiochemical characteristics of experimental farm.

Year	Soil texture	Bulk density	Sand (%)	Silt (%)	Clay (%)	N (mg/kg)	P (mg/kg)	K (mg/kg)	C (%)	EC (ds/m)	pH
2014	Sandy-loam	1.6	51	28	21	0.06	14.6	185	0.76	0.95	8.1
2015	Clay-loam	1.4	31	29	40	0.08	13.5	180	0.69	1.1	7.8

and end of each experimental plot as the margin effect, samples were randomly taken from 10 plants pereach plot. Morphological traits (plant height, leaf area, number of sub-branches and plant dry weight) were measured at full flowering stage. These plants were taken from the depth of 5 cm of the soil surface and after counting the number of sub-branches, leaveswere isolated in order to measure their area with EE540-012. Leaves, along with plant remains were dried in the oven at 70 °C for 48 h and plants dry weight was measured with an accuracy of 0.01 g.

Irrigation treatments

The plants in all experimental plots were watered uniformly when 30% of available soil water (ASW) was depleted for 25 days, then when the plants were approximately 15 cm tall irrigation regimes were applied and continued until plants reached 60-70% flowering stage. All experimental operations were repeated in the second year (*i.e.*, 2015).

The amount of water stored in the root zone of the soil between field capacity and the permanent wilting point is described as ASW, which can be easily used by plants. ASW was calculated based on following equation:

$$ASW = (W_{FC} - W_{WP}) \times Bd \times V$$

Where W_{FC} and W_{WP} are the gravimetric soil-water content (%) at field capacity (FC) and permanent wilting point (WP), respectively, Bd is the bulk density value of the soil (g cm^{-3}) and V is the volume of soil layer in the root zone depth (m^3). The mean root zone depth of dragonhead plants was designated as 35 cm.

Readily available soil water (RAW) refers to the fraction of ASW that a plant can readily uptake from the entire root zone without enduring consequences drought stress, was measured according to the following equation.

$$RAW = p \times ASW$$

The *p* factor normally differs for various plants ranging from 0.3 for shallow-rooted plants at high rates of evapotranspiration, ETc (> 8 mm day^{-1}) to 0.7 for deep-rooted plants at low rates of ETc (< 3 mm day^{-1}). The factor *p* was applied to measure the required time of irrigation to avoid drought stress. A value of 0.50 for *p* is commonly used for many plants. Therefore, the latter value was used for *D. moldavica* L. in this study. The fraction p is defined as evaporation power of the atmosphere based on the following equation:

$$p = p_{rec} + 0.04(5-ET_c)$$

Where p_{rec} is the recommended value for many field crops, and *p* is the adjusted value for atmospheric evaporative demand. Subsequently, the watering treatments were scheduled according to the maximum allowable depletion (MAD) percentage of

ASW in root zone [21]. In order to determine soil water content a TDR probe was used two days after irrigation and continued up to one day before next irrigation event.

The depletion of ASW content related to the soil water potential was calculated by a soil moisture release curve. The volume of irrigation water (V_{irrig}) required to increase the root zone soil water to FC was determined by following equation:

$$V_{irrig} = \frac{ASW \times f}{Ea}$$

Where f is the depletion fraction of ASW from the root zone depth, and Ea is the irrigation efficiency (%), which was supposed to be 70% entire the growing season [22]. The irrigation water was distributed by a system of pipes and the volume was measured by a flow meter.

Essential oil extraction and analysis procedure

The essential oil of samples (30 g in three replications) was isolated by hydro-distillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia [23]. Essential oil percentage (EP) of the samples was calculated in terms of dry weight of the plant materials (w/w) according to the following formula:

$$\text{EP (\%)} = \left(\frac{\text{Essential oil weight}}{\text{Plant sample dry weight}}\right) \times 100$$

However, the essential oil yield (EY) was obtained as EP × plant dry weight yield divided by 100 (kg/ha). The isolated oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 ◦C until analysis.

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was carried out on fused silica capillary DB-5 column (30 m×0.25 mm i.d.; film thickness 0.25 µm). The injector and detector temperatures were kept at 250 ◦C and 300 ◦C, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.1 mL/min; oven temperatureprogram was 60–250 ◦C at the rate of 4 ◦C/min and finally held isothermally for 10 min; split ratio was 1: 50. GC–MS analysis was carried out by use of Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column(60 m×0.25 mm i.d.; film thickness 0.25 µm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 200 ◦C and 250 ◦C, respectively. Mass range was from 35 to 456 amu. Oven temperature program was the same asmentionned above for the GC.

Identification of compounds

The constituents of the essential oils were identified by calculating their retention indices under temperature-programmed conditions for n-alkanes (C6–C24) and the oil on a DB-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparing their mass spectra with those of the internal reference mass spectra library (Adams and Wiley 7.0) or with authentic compounds and confirmed by comparingtheir retention indices with authentic compounds or with those of reported in the literature [24]. For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

Statistical analysis

The data set was first tested for skewness and kurtosis. Appropriate transformation was applied for specific characters that showed non-normal distributions. Data were subjected to analysis of variance (ANOVA) and comparison of means.These analyses were conducted using the MSTAT-C and SPSS 16.0 statistical software.

Results

Combined analysis of the data given in Table 2 reveals that drought stress, zeolite and bio-fertilizers application had significantly affectes on studied traits in both years.

Mean comparisons showed that the effect of employed treatments were significant ($P<0.05$) on some morphological traits (Table 3). The highest amount of plant height, leaf area, dry matter, number of ordinary branches and FTBY obtained through 90%FC. Maximum essential oil percentage and yield were obtained in 30% and 60% FC, respectively. The lowest values of these parameters were obtained in 30%FC. The minimum percentage of essential oil was produced in 90% FC. In other hand, treatment of 30%FC caused the lowest value of FTBY. Application of zeolite improved plant height, number of ordinary branches, essential oil yield and FTBY but it had no affect on leaf area, dry matter and essential oil percentage. The combination of nitroxine+phosphate barvar-2 gave the highest values of these parameters compared to the untreated plants (control). On the other hand, distinct application of these bio-fertilizers improved all parameters. In spite of all that, there were no significant differences between it and combination of nitroxine+phosphate barvar-2 in some characters. Interaction effects of employed treatments on morphological characteristics and essential oil production of dragonhead in two years was presented in Table 4.

Application of zeolite had the highest effect on plant height, leaf area, dry matter and FTBYin 90%FC and essential oil percentage and yield in 60%FC (Table 5). Similarly, combination of nitroxine+phosphate barvar-2 caused maximum value of plant height, leaf area, dry matter and FTBY in 90%FC and essential oil percentage and yield in 60%FC (Table 5). Also, application of zeolite accompanied with combination of nitroxine+phosphate barvar-2 produced the highest value of characters (Table 6).

Essential oil constituents

Triple interaction effects of drought stress, bio-fertilizers and zeolite on morphological traits and essential oil production of dragonhead was shown in Table 7. Also, Combined analysis of variance was presented in Table 8. The results indicated that drought stress, zeolite and bio-fertilizer had significant effects on studied traits in both years. The zeolite had no significant effect on essential oil components including Geranio, Geranial, non ester combinations (Neral+ Geraniol+ Geranial); and ester combinations (Neryl acetate+ Geranyl acetate).

Table 2- Analysis of variance (ANOVA) for morphological characteristics and essential oil production of D. *moldavica* under experimental treatments

Source of variations	df	MS						
		PH	LA	DM	LBN	EP	FTBY	EY
Year	1	0.168ns	765.444ns	0.163*	34.028**	0.002*	61167.361ns	0.006ns
Rep (Year)	2	6.739ns	924.389	0.023ns	0.424ns	0.001ns	9353.576ns	0.250ns
A	2	2292.372**	920549.396**	48.236**	26.340**	0.051**	51294166.896**	169.685**
Year×A	2	25.337*	1372.299ns	0.052ns	0.965ns	0.049**	67138.215ns	31.812**
Main Error	8	5.438	1488.483	0.021	0.684	0.001	16170.983	0.102
B	1	258.459**	50400.250**	0.162*	0.444ns	0.001*	810000.000**	0.957*
Year×B	1	4.594ns	3700.694*	0.020ns	4.694**	0.001ns	191844.000**	0.137ns
A×B	2	192.911**	35336.021**	0.133**	5.215**	0.001ns	510846.271**	1.198**
Year×A×B	2	2.347ns	37.674ns	0.150**	0.674ns	0.001ns	49148.313**	0.350ns
C	3	5415.179**	943546.833**	13.239**	94.630**	0.002**	175572.720**	13.365**
Year×C	3	4.938ns	478.500ns	0.094*	7.213**	0.000ns	16273.484**	0.536*
A×C	6	105.817**	3838.813**	1.297**	0.942ns	0.001*	32442.537**	2.116**
Year×A×C	6	3.061ns	1266.993ns	0.024ns	0.678ns	0.000ns	3534.352ns	0.478*
B×C	3	6.017ns	205.157ns	0.380**	0.630ns	0.001ns	15230.613**	0.075ns
Year×B×C	3	5.618ns	772.380ns	0.025ns	0.620ns	0.000ns	6276.359ns	0.282ns
A×B×C	6	14.754**	3131.623**	0.178**	0.206ns	0.001ns	15230.613**	0.366*
Year×A×B×C	6	3.127ns	698.664ns	0.038ns	0.294ns	0.001ns	6276.359ns	0.224ns
Secondary Error	84	3.043	978.967	0.027	0.581	0.001	2897.982	0.163
CV (%)		3.34	1.90	3.49	9.60	7.95	1.61	5.92

† A: Drought stress; B: Zeolite; C: Bio-fertilizer.

†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield.

††† ns, *, **: Non-significant, significant at 0.01% and 0.05% probability levels, respectively.

Table 3- Mean comparison of drought stress, bio-fertilizers and zeolite effects on morphological characteristics and essential oil production of dragonhead in two years (2014 and 2015).

Treatment	PH (cm)	LA (cm^2)	DM (g)	LBN (No.)	EP (%)	FTBY (kg/ha)	EY (kg/ha)
Year							
1	52.201a	1.646a	4.694b	8.431a	0.211a	3.366a	6.805a
2	52.269a	1.641a	4.761a	7.458b	0.204b	3.323b	6.818a
A							
1	58.525a	1.759a	5.555a	8.708a	0.170b	4.141a	7.093b
2	53.343b	1.681b	5.015b	7.896b	0.222a	3.716b	8.535a
3	44.837c	1.490c	3.613c	7.229b	0.230a	2.177c	4.806c
B							
1	50.896b	1.625a	4.694a	8.000a	0.210a	3.270b	6.730b
2	53.575a	1.663b	4.761b	7.889a	0.204b	3.420a	6.893a
C							
1	36.300d	1.418c	3.844b	5.528b	0.199a	3.096b	6.063b
2	59.553b	1.744a	4.841a	8.750a	0.207a	3.383ab	6.826ab
3	49.242c	1.638b	5.028a	8.528a	0.208a	3.339ab	6.801ab
4	63.847a	1.775a	5.197a	8.972a	0.215a	3.561a	7.555a

† A: Drought stress (1, 2 and 3 refer to irrigation after 30, 60 and 90% depletion of available soil water, respectively); B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).

†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield.

†††Means in each column followed by the same letter are not significantly different ($P < 0.05$).

Table 4- Interaction effects of drought stress, bio-fertilizers and zeolite on morphological characteristics and essential oil production of dragonhead in two years (2014 and 2015).

Treatment	PH (cm)		LA (cm^2)		DM (g)		LBN (No.)	
	Year		Year		Year		Year	
	1	2	1	2	1	2	1	2
A								
1	57.83b	59.22a	1756a	1764a	5.489b	5.620a	9.083a	8.333b
2	53.19c	53.50c	1687b	1675b	4.979c	5.051c	8.333b	7.458c
3	45.58d	44.09e	1495c	1486c	3.613d	3.612d	7.875bc	6.583d
B								
1	50.68b	51.11b	1622c	1628c	4.365b	4.739a	8.306a	7.694b
2	53.72a	53.43a	1670a	1655b	4.739a	4.783a	8.556a	7.222c
C								
1	36.44d	36.16d	1418d	1417d	3.833d	3.856d	5.944e	5.111f
2	59.50b	59.61b	1747b	1742b	4.813c	4.868c	9.889a	7.611d
3	49.56c	48.93c	1645c	1630c	5.038b	5.017b	8.722bc	8.333c
4	63.30a	64.39a	1775a	1776a	5.090b	5.303a	9.167b	8.778bc

Table 4- Continue

Treatment	EP (%)		FTBY (kg/ha)		EY (kg/ha)	
	Year		Year		Year	
	1	2	1	2	1	2
A						
1	0.173d	0.168d	4149a	4134a	7.218c	6.969d
2	0.202b	0.258a	3780b	3652c	7.656b	9.413a
3	0.258a	0.186c	2170d	2183d	5.540e	4.072f
B						
1	0.214a	0.207b	3255d	3285c	6.692b	6.767ab
2	0.208b	0.201c	3478a	3362b	6.917a	6.869ab
C						
1	0.203b	0.194c	3128e	3063f	6.173d	5.952d
2	0.201b	0.204b	3420c	3346d	6.821bc	6.862bc
3	0.208b	0.208b	3326d	3352d	6.626c	6.976b
4	0.222a	0.208b	3592a	3531b	7.598a	7.512a

† A: Drought stress (1, 2 and 3 refer to irrigation after 30, 60 and 90% depletion of available soil water, respectively); B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).
†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield.
††† Means in each column followed by the same letter are not significantly different ($P < 0.05$).

Table 5- Interaction effects of drought stress, zeolite and bio-fertilizers on yield and morphological characteristics of dragonhead

Treatment	PH (cm)			LA (cm^2)			DM (g)			LBN (No.)		
	A			A			A			A		
	1	2	3	1	2	3	1	2	3	1	2	3
B												
1	58.47a	23.03b	41.19d	1764a	1669c	1442e	5.500b	5.041c	3.540e	9.125a	7.875bc	7.000d
2	58.58a	53.63b	48.49c	1755a	1693b	1539d	5.609a	4.988c	3.685d	8.292b	7.917bc	7.458c
C												
1	42.65h	34.17i	32.08j	1540h	1435j	1279k	4.401e	3.882f	3.249h	6.083e	5.583e	4.917f
2	67.53b	63.16c	47.97g	1853b	1804c	1576g	5.795b	5.053d	3.674g	9.417b	8.917bc	7.917d
3	53.72e	51.63f	42.38h	1762d	1661e	1490i	5.807b	5.510c	3.766fg	9.167bc	8.500cd	7.917d
4	70.21a	64.42c	56.92d	1885a	1824c	1617f	6.216a	5.613c	3.761fg	10.170a	8.583cd	8.167d

Table 5- Continue

Treatment	EP (%)			FTBY (kg/ha)			EY (kg/ha)		
	A			A			A		
	1	2	3	1	2	3	1	2	3
B									
1	0.168d	0.234b	0.228ab	4129a	3522c	2158e	6.987c	8.309b	4.893d
2	0.172d	0.236a	0.215c	4154a	3910b	2195d	7.199c	8.760a	4.719d
C									
1	0.152f	0.230ab	0.215c	3807d	3391g	2089j	5.870g	7.814d	4.504i
2	0.167e	0.231ab	0.223bc	4205b	3758e	2185i	6.993f	8.636b	4.850h
3	0.174e	0.225ab	0.225ab	4194b	3643f	2179i	7.370e	8.189c	4.843h
4	0.188d	0.233a	0.225ab	4359a	4072c	2253h	8.139cd	9.499a	5.027h

† A: Drought stress (1, 2 and 3 refer to irrigation after 30, 60 and 90% depletion of available soil water, respectively); B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).
†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield.
††† Means in each column followed by the same letter are not significantly different ($P < 0.05$).

Table 6- Interaction effects of zeolite and bio-fertilizers on morphological characteristics and essential oil production of *D. moldavica* L.

Treatment	PH (cm)		LA (cm^2)		DM (g)		LBN (No.)	
	B		B		B		B	
	1	2	1	2	1	2	1	2
C								
1	34.57b	38.03g	1398g	1437f	3.776f	3.913e	5.444c	5.611c
2	57.93f	61.17c	1725c	1764b	4.955c	4.727d	8.722ab	8.778ab
3	48.09d	50.39e	1617e	1658d	4.971bc	5.084b	8.667ab	8.389b
4	62.99h	64.71a	1760b	1791a	5.073b	5.320a	9.167a	8.778ab

Table 6- Continue

Treatment	EP (%)		FTBY (kg/ha)		EY (kg/ha)	
	B		B		B	
	1	2	1	2	1	2
C						
1	0.201cd	0.197d	3023g	3168f	5.999c	6.127c
2	0.207bc	0.207bc	3328d	3443c	6.710b	6.943b
3	0.211b	0.205bc	3221e	3457c	6.680b	6.922b
4	0.221a	0.209b	3512b	3611a	7.530a	7.580a

B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).
†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield.
††† Means in each column followed by the same letter are not significantly different ($P<0.05$).

Table 7- Triple interaction effects of drought stress, bio-fertilizers and zeolite on morphological traits and essential oil production of dragonhead.

Treatment		PH (cm)			LA (cm^2)			DM (g)			LBN (No.)		
		A			A			A			A		
B	C	1	2	3	1	2	3	1	2	3	1	2	3
1	1	41.88hi	33.65k	28.17l	1574j	1413l	1234n	4.312g	3.830hi	3.185k	6.333h	5.333ij	4.667j
1	2	67.72b	62.70cd	43.38gh	1860ab	1790def	1524j	5.842c	5.427e	3.597j	9.667b	9.000bcde	7.500g
1	3	52.35f	51.72f	40.22i	1759f	1672g	1422kl	5.768cd	5.420e	3.725hij	9.667b	8.500cdefg	7.833fg
1	4	71.92a	64.06c	52.98f	1891a	1802de	1588i	6.078b	5.488e	3.653ij	10.83a	8.667bcdef	8.000efg
2	1	43.42gh	34.69jk	36.00j	1532j	1457k	1323m	4.490g	3.935h	3.313k	5.833hi	5.833hi	5.167ij
2	2	67.33b	63.62c	52.57f	1846bc	1819cd	1628h	5.748cd	4.680f	3.752hij	9.167bcd	8.833bcdef	8.333defg
2	3	55.08e	51.55f	44.53g	1765ef	1650gh	1558ij	5.845c	5.600de	3.807hi	8.667bcdef	8.500cdefg	8.000efg
2	4	68.50b	64.77c	60.85d	1879ab	1846bc	1646gh	6.353a	5.738cd	3.868h	9.500bc	8.500cdefg	8.333defg

Table 7- Continue

Treatment		EP (%)			FTBY (kg/ha)			EY (kg/ha)		
		A			A			A		
B	C	1	2	3	1	2	3	1	2	3
1	1	0.153k	0.235abc	0.215fg	3788d	3209g	2072k	0.153k	7.667ef	4.462i
1	2	0.167j	0.230bcde	0.225bcdef	4216b	3585e	2168ij	0.167j	8.323cd	4.810i
1	3	0.172ij	0.233abcd	0.228bcde	4162b	3351f	2151ij	0.172ij	7.918de	4.887i
1	4	0.182i	0.237ab	0.245a	4349a	3944c	2243h	0.182i	9.327ab	5.415h
2	1	0.150k	0.225bcdef	0.215fg	3826d	3573e	2105jk	0.150k	7.962cde	4.547i
2	2	0.167j	0.232bcd	0.220def	4195b	3931c	2203hi	0.167j	8.948b	4.890i
2	3	0.177ij	0.217efg	0.222cdef	4227b	3936c	2207hi	0.177ij	8.460c	4.800i
2	4	0.193h	0.230bcde	0.205g	4369a	4200b	2263h	0.193h	9.672a	4.638i

† A: Drought stress (1, 2 and 3 refer to irrigation after 30, 60 and 90% depletion of available soil water, respectively); B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).

†† PH: Plant height; LA: Leaf Area; DM: Dry Matter; LBN: Lateral Branches Number; EP: Essential oil Percentage; FTBY: Flowering Top Branches Yield; EY: Essential oil Yield. Means in each column followed by the same letter are not significantly different ($P < 0.05$).

Table 8- The analysis of variance (ANOVA) for major essential oil constituents in D. *moldavica* under experimental treatments

SOV	df	Mean square				
		Neral	Geraniol	Geranial	Neryl acetate	Geranyl acetate
Year	1	2698.456**	1218.708**	9077.484**	36803.225**	106018.074**
Rep(Year)	2	0.454	0.801ns	2.181ns	5.352	5.768*
A	2	484.758**	285.652**	25.072**	1118.145**	14.325**
Year×A	2	224.110**	20.035**	66.616**	616.737**	1581.034**
Main Error	8	1.601	0.419	1.994	6.543	1.104
B	1	1.690*	0.299ns	0.343ns	1.509ns	1.902ns
Year×B	1	3.680**	0.139ns	0.133ns	0.360ns	3.395ns
A×B	2	1.230ns	0.081ns	0.983ns	0.129ns	1.273ns
Year×A×B	2	0.982ns	0.239ns	0.894ns	1.978ns	4.348ns
C	3	43.587**	85.258**	545.229**	1093.353**	23.420**
Year×C	3	13.646**	1.392ns	229.121**	333.445**	82.229**
A×C	6	7.197**	6.548**	19.694**	34.669**	4.397*
Year×A×C	6	4.553**	1.549*	26.584**	30.055**	19.805**
B×C	3	2.749**	0.389ns	1.676ns	5.169*	1.936ns
Year×B×C	3	2.569**	0.114ns	1.167ns	3.453ns	0.350ns
A×B×C	6	1.226**	1.060ns	1.191ns	4.978**	1.082ns
Year×A×B×C	6	1.106*	1.258*	2.337**	2.838ns	1.126ns
Secondary Error	84	0.403	0.562	0.754	1.459	1.663
CV(%)		6.69	7.73	5.52	3.55	3.86

† A: Drought stress; B: Zeolite; C: Bio-fertilizer.
†† *: Significant in 0.01%; **: Significant in 0.05%; ns: no significant.

With increasing drought intensity the flower yield was significantly decreased. The essential oil percentage was increased by water deficit, so that with increasing drought intensify from 90%FC to 30%FC essential oil content was reached from 0.170% to 0.230%, but there was no significant different between 30%FC and 60%FC means. Also, the highest oil yield reached from 60%FC.

Mean comparison of zeolite application indicated that the highest yield of flowers belonged to 2 g zeolite. Zeolite application hasn't had significant effect in essential oil percentage so that with application of zeolite, essential oil content was decreased from 0.210% to 0.204%. Also, use of 2 g zeolite in 1kg soil had more effective essential oil yield than control.

Analysis of interaction effects showed that flower yield increased in all levels of field capacity with increasing zeolite from 0 to 2 g. Combination of nitroxin and phosphate barvar-2 produced the highest yield of flower in all levels of water deficit (Table 9). Also, combined application of bio-fertilizers caused the maximum flower yield in presence of zeolite (Table 9). At the completion of the double interactions, the maximum amount of flower yield achieved from application of 2 g zeolite and combination of nitroxin and phosphate barvar-2 in 90% FC (Table 9). The highest essential oil percentage was observed by 30%FC in absent of zeolite and combined application of nitroxin and phosphate barvar-2 (Table 9). The maximum essential oil yield was produced in 2 g zeolite and 60%FC, nitroxin×phosphate barvar-2 and 60% FC and finally 2 g zeolite and nitroxin×phosphate barvar-2 and 60%FC (Table 9). Combination of nitroxin and phosphate barvar-2 were showed maximum percentage of essential oil and yield of flowers and essential oil. Results indicated that these traits increased generally over control by using all fertilizer treatments. However, the highest values wereobtained by the combined treatment of nitroxin+phosphate barvar-2 in both years with none significant differences between them in most cases. On the other hand, the lowest values of these parameters were produced by the control.

Data presented in Table 10 indicated that the GC-MS analysis of the essential oil identified 14 compounds. In this case, the main constituents of the essential oil of dragonhead were including of two large groups called ester and non ester combinations.The main compounds of ester combinations were Geranyl acetate and Neryl acetate and the main compounds of non ester combinations were Neral, Geraniol and Geranial. The components of essential oil vary slightly from year to year. This may be mostly due to changes in climate conditions and the effect of climate on chemo types of plants. In this study, Neral, Geraniol and Geranial were remarkably produced at 2014 and Neral, Geraniol, Geranial, Geranyl acetate and Neryl acetate were mainly produced at 2015.

The results of mean comparisons indicated that the maximum percentage of ester and non ester combinations produced in 30% FC and 60-90% FC, respectively. Also, application of 2 g zeolite and combination of nitroxin and phosphate barvar-2 increased composition values in this experiment, separately. There was inverse relation between ester and non ester combinations, so that increase of Geranyl

Table 9- Mean comparison of the effect of drought stress, bio-fertilizers and zeolite on major essential oil constituents of *D. moldavica* in two years

Treatment	Neral (%)	Geraniol (%)	Geranial (%)	Neryl acetate	Geranyl acetate	NEC (%)	EC (%)
Year							
1	13.820	12.603	23.661	-	-	50.038	6.291
2	5.162	6.785	7.781	3.50	57.06	18.064	60.558
A							
1	9.419b	12.220a	15.356a	3.73a	57.90b	36.348a	32.928a
2	12.705a	9.501b	16.553a	3.48b	62.46a	37.300a	33.336a
3	6.350c	7.352c	15.253a	3.29b	50.82c	28.505b	34.010a
B							
1	9.383b	9.648a	15.769a	3.50a	57.09a	33.949a	33.309a
2	9.599a	9.739a	15.672a	3.49a	57.03a	34.154a	33.539a
C							
1	8.021a	8.069b	11.775b	3.31b	54.22c	26.600b	32.243a
2	9.484a	10.842a	14.003b	3.44ab	56.71b	33.369ab	33.638a
3	9.794a	8.700b	19.470a	3.59a	58.69a	37.104a	34.054a
4	10.666a	11.164a	18.337a	3.65a	58.62a	39.131a	33.763a

† A: Drought stress (1, 2 and 3 refer to irrigation after 30, 60 and 90% depletion of available soil water, respectively); B: Zeolite (1, 2 refer to 0 and 2 g/kg, respectively); C: Bio-fertilizers (1, 2, 3, and 4 refer to control, nitroxin, barvar-2 and nitroxin×barvar-2, respectively).
†† NEC: Non Ester Combinations (Neral+ Geraniol+ Geranial); EC: Ester Combinations (Neryl acetate+ Geranyl acetate).
††† Means in each column followed by the same letter are not significantly different ($P < 0.05$).

Table 10-The chemical constituents of essential oil in dragonhead plants under 60%FC with application of zeolite and nitroxin+phosphate barvar-2 in 2014 and 2015

	2014			2015	
Components	**RT**	**(%)**	**Components**	**RT**	**(%)**
n-decanal	772	1.6	*E*-β-ocimene	1039	0.8
n-nonanal	527	3.2	Thymol	1292	1.1
p-cymene	315	4.1	*Transe*-limonene oxide	1154	2.7
Methslgranate	1071	2.2	Methylgranate	1322	0.5
Linalool	378	1.9	Linalool	1104	3.4
Caryophyllene oxide	1698	3.7	Caryophyllene oxide	1568	1.2
Thymol	996	2.8	*Cis*- limonene oxide	1145	0.7
Neral	863	13.82	Neral	1249	5.16
Carvacrol	1018	4.1	Geranyl acetate	1373	57.06
Geraniol	900	12.56	Geraniol	1263	6.78
Geranial	943	23.66	Geranial	1280	7.78
Piperiton	887	3.6	Nerol	1233	1.8
Neryl acetate	1233	6.29	Neryl acetate	1352	3.50
Spathulenol	1687	3.8	Spathulenol	1568	1.6
Neral+ Geraniol+ Geranial		50.04			19.72
Neryl acetate+ Geranyl acetate		6.29			60.56
Total identified		87.33			94.08

RT: Retention time

acetate (ester combination) caused perceptible decrease in non ester combinations like Neryl, Geraniol and Geranial at second year. As regard that the trend of these compositions changes affected by treatments of zeolite and bio-fertilizers was similar, probably it occurred due to water deficit treatments. As mentioned, ester combinations in 30% FC and non ester combinations in high water availability (60 and 90%FC) achieved the maximum values. It reflects the effect of environmental conditions, especially moisture availability on the amount and composition of dragonhead. Generally, the main compositions didn't change due to zeolite and bio-fertilizers, but it substantially changed by different levels of drought stress. In this case, increasing of some compositions caused decreasing in remains. It seems plant in face of different condition environmental increase some compositions in oil and reduction others that it has direct and indirect relation.

Discussion

The results of variance analysis showed that the drought stress significantly affected the morphological and yield traits such as plant height, leaf area, dry matter, LBN, oil percentage, FTBY and oil yield. These results about significant effects of water stress, zeolite and fertilizers application on morphological and yield parameters were similar to other finding such as: dragonhead [25-28].

Mean comparison for drought levels revealed that the highest mean in all traits except oil percentage belonged to irrigation in 90%FC. In other words, almost all traits and essential oil yield were reduced with increasing the drought level from 90%FC to 30%FC but with increasing drought intensity from 90%FC to 30%FC the essential oil percentage was increased. Reduction in essential oil yield may be due to disturbance in photosynthesis carbohydrate production under stress condition and suppuration of the plant growth [29]. Three mechanisms decrease yield of aromatic plants affected by drought stress condition: a) by reducing whole canopy absorption of incident photosynthetic active radiation; b) by reducing dry matter production through influence on absorbed photosynthetic active radiation; c) by reducing the harvest index. Badalzadeh et al. [30] reported that the minimum and maximum value of morphological characteristics (plant height, number of ordinary branches and dry matter) and essential oil yield of dragonhead were obtained by 50%FC and 100%FC. Garosi et al. [31] described that increasing drought stresses caused reduction both in fresh and dry weight Hyssop such as LA, DM, PH, however, with increasing drought intensity from 70%FC to 30%FC the essential oil percentage was increased. Also, the highest amount of PH, FTBY and FY produced in desired irrigation [32]. They explained that beginning drought stress reduced value of morphological parameters specially PH. The results of effect of drought in medicinal plants indicated that water deficit during the vegetative can result in shorter plants, smaller leaf areas and least leaves of dragonhead [28], Marigold [33], Coriander [34] and Mint [35].

In this experiment the highest value for oil percentage was observed in 30%FC and the highest shoot yield belong to 90%FC but the highest oil yield was observed in 50%FC.It's similar with finding of Amiri et al. [36]. It seems that the drought stress increases the essential oil percentage of more medicinal and aromatic plants, because in case of stress, more metabolites are produce in the plants and substances prevent from oxidization in the cell, but essential oil yield reduce under drought stress, because the interaction between the amount of the essential oil percentage and shoot yield its consider important as two components of the essential oil yield an therefore irrigation in 50%FC is suitable point for having balance between oil percentage and shoot yield that lead to the highest essential oil yield [34].

Also, our results showed that the effect of zeolite levels was significant for all traits except for number of ordinary branches. Zeolite application has had different effect on growth parameter and oil yield. Application of zeolite improved plant height, number of ordinary branches, essential oil yield and FTBY but it had no affect on leaf area, dry matter and essential oil percentage. Positive effect of zeolite reported in other plants like rice [37] and Jinkgo [38]. Similar to our results, zeolite application has significant affect on DM, PH and EP and increased dragonhead essential oil yield [27]. They believed non significant effects of zeolite on some parameters werecaused insufficient soil structure and/or overusing. Effects of zeolite were reported by other researchers could be are result of high potentially to absorb water and conserve water in the soil and improve physical properties [39].

Also bio-fertilizer application had significant effect on all traits. In this case, incorporation of nitroxine and phosphate barvar-2 increased morphological characteristics and traits related to essential oil yield more than other treatments. Similar to these results, increasing effect of nitrogen and phosphate fertilizers on morphological traits (e.g., PH, LA, DM, number of flowering and ordinal branches) and parameters related to yield reported on various plants such as dragonhead [40], melisa [41], fennel [42], plantain [43], rosemary [44], oregano [45], thyme [46], camomille [47]. Pouryousef et al. [48] believed application of nitrogen fertilizers and its resonant effect on growth and cell divisions caused increase PH. Darzi et al. [49] reported that the minimum values of morphological traits and subsequent yield produced through non fertilizer application (control). Nitroxine application increased PH, essential oil percentage and yield of marigold [32]. In other case, Alijani et al. [49] indicated that phosphate fertilizer was main causing of PH, DM, NB, FTBY, essential oil percentage and yield. These results are similar to finding of Praszna and Bernath [50], Valadabadi et al. [51]. Fertilizers increase essential oil yield of aromatic plants by improving photosynthesis, chlorophyll, activity of rabisco enzyme and leaf growth [31, 52].

In order to more investigate, interaction effects at water deficit, zeolite and bio-fertilizers were calculated. Results of interaction effects were similar with the reports of Hendawy et al. [53] and Badalzadeh et al. [30]. High content of polymer with water supply caused opening stomata for a long time, sub squinty good fixation of CO_2 resulted an increase of dry matter in medicinal plant [54]. With regard to Table 7, it can be seen that effect of water deficit×zeolite×bio-fertilizers had significant different on all traits except LBN and EP. The highest essential oil yield was observed in 60%FC with 2 g zeolite with combined application of nitroxin+phosphate barvar-2. Interaction effect between zeolite and water deficit caused significant reduction in drought intensity on growth parameter. Zeolite application increased the water use efficiency. In other word, zeolite application decreased the adverse effect of stress and caused to increase WUE, effect of water stress on WUE depends on plant species, phonological stage of plant in drought period and stress intensity. Also, nitroxine and phosphate barvar-2 improve fertilization of soil, nutrition availability and absorption efficiency; finally these integrative systems caused high effect on morphological traits and essential oil yield.

The results about significant effects of water stress, zeolite and fertilizers application on essential oil yield were similar to other finding such as dragonhead [26, 28].

It seems that the drought stress increases the essential oil percentage in aromatic plants due to producing of more metabolites in the plants for preventing roll from oxidization in the cell. In other hand, essential oil yield reduce under drought stress due to the interaction between the amount of the essential oil percentage and flower yield. Its consider important as two components of the essential oil yield therefore irrigation in 60%FC is suitable point for having balance between essential oil percentage and flower yield that lead to the highest essential oil yield. Badalzadeh et al. [41] reported that the minimum and maximum value of essential oil yield of dragonhead was obtained by 50%FC and 100%FC.

Similar to our results, Zeolite application has significant effect on EP and increased dragonhead EY [27].

Results of interaction effects were similar with the reports of Hendawy et al. [53] and Badalzadeh et al. [43]. Interaction effect between zeolite and water deficit caused significant reduction in drought intensity on plant. Zeolite application increased the water use efficiency. In other word, zeolite application decreased the adverse effect of stress and caused to increase WUE. Also, nitroxine and biophosphate improve fertilization of soil, nutrition availability and absorption efficiency; finally these integrative systems caused high effect on morphophysiological traits and followed in essential oil yield.

In the present study the highest values were gained by the combined treatment of nitroxin+phosphate barvar-2 in both seasons with none significant differences between them in most cases. On the other hand, the lowest values of these parameters were produced by the control. These results are in agreement with those reported by Rahimzadeh et al. [55] and Yousefzadeh et al. [40] on dragonhead; Barandozi and Pourmaleknejad [56] on thymus; Abd El-Wahab [57] on origanum and sinaicum and El-Khyat [58] on rosmarinus. Furthermore, Mohamed et al. [59] showed that *Ocimumbasilicum* L. cv. Genovese which received bio-fertilizer showed the highest significant increase of essential oil yield.

In this study, biosynthesis of Neral, Geraniol, Geranial at 2014 and Neral, Geraniol, Geranial, Geranyl acetate and Neral acetate at 2015 were similar to findings of Darzi et al. [60] and Yousefzadeh et al. [40], who stated that the analysis ofessential oil composition of the dragonhead revealed the presence of 16 identified compounds, representing 90% of the essential oil with Neral, Geraniol, Geranial, Neryl acetate and Geranyl acetate being the main components. The results about changes of compositions of essential oil upon employed treatments are agreement with Argyropoulou et al. [61] and Santos-Gomes et al. [62] reports. They believed that the different observations in quality and quantity of essential oil compositions in aromatic and medicinal plants occurred with many factors such as origin, ecological elements, genetic variation, different methods applied in cultivation and etc. In this case, Ahmadian et al. [63] reported that increasing of cumin aldehyde had direct association with increasing of para cymene and indirect association with increasing of α-pinene, β-pinene and γ-terpinene. They proposed that this relationshiprevealed a complementary relation among oil compositions.

Conclusion

The results exhibit that the biofertilizers and phosphate barvar-2 had significantly effect quantitative and qualitative production of *D. moldavica* L. under different drought stress conditions. From the results obtained it can be cancluded that the effect of water deficit × zeolite × biofertilizers had significant effects on most of the examined traits. Also, the highest essential oil yield was observed in 60%FC with 2.5 g zeolite with combined application of nitroxin+phosphate barvar-2. Thus, the interaction effect between zeolite and water deficit caused significant reduction in drought intensity influence on growth parameter of plants.

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