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Original Article

Physiological, Biochemical and Antioxidant Responses of Bakhtiari Savory (*Satureja bachtiarica* Bunge.) to Manure Application and Plant Density under Dryland Farming Condition

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Abstract

The use of agricultural practices such as plant density and environmental friendly fertilizers are effective in improving plant products. This study was conducted to determine the physiological and biochemical response of Bakhtiari savory (*Satureja bachtiarica* Bunge.) to cattle manure (30-ton ha^{-1}) and plant density (high plant density, HPD: 80000, medium plant density, MPD: 40000, and low plant density, LPD: 26666 plant ha^{-1}) during 2018 and 2019. Chlorophyll (Chl) content, relative water content (RWC), Malondialdehyde (MAD), proline concentrations, total phenol content (TPC), total flavonoid content (TFC), and essential oil (EO) percentage of Bakhtiari savory were measured under dryland farming condition. Chl a and b increased under manure application at high/MPD in the second year. RWC at high/MPD with manure application in the second-year plants was higher compared to other experimental treatments. The highest MAD was observed in plants at LPD with non-manure application in the first year. Increased proline concentration was found at LPD compared to MPD and HPD. TPC and TFC in second-year plants were significantly greater than the first-year plants. Organic fertilizer improved TPC and TFC content. The greatest TPC and TFC were observed at MPD with manure application in the second-year plants. EO percentage in MPD was higher than other plant densities. The highest EO percentage was achieved at MPD with manure application in the second-year plants. It could be suggested that use of cattle manure and HPD or MPD leads to obtain higher plant production.

Keywords: Essential oil, Chlorophyll, Flavonoid, Proline, Organic fertilizer.

Introduction

The genus *Satureja* (Lamiaceae family) consists of 200 species of herbs and shrubs, mainly distributed in the Mediterranean area [1]. This genus contains 16 species in Iran with nine endemic species, which are commonly distributed in rocky mountainous areas [2]. Bakhtiari savory (*Satureja bachtiarica* Bunge.), locally named "Merzeh Koohi", is well-known medicinal and aromatic herb. It is traditionally used as an analgesic and antiseptic agent and the phytochemical studies have shown that essential oil (EO) of Bakhtiari savory has antifungal and antibacterial properties [2].

Recently, the strategies of alleviating the chemical fertilizers with applying non-chemical materials are discussed to obtain the high-quality products [3, 4]. In arid and semi-arid regions like most parts of Iran the soil nutrients are low. Therefore, the soil reinforcement with fertilizer allows producing and developing the sustainability of crop production [5-7]. Nowadays, the use of organic fertilizers instead of chemical materials is the main purpose in producing the agricultural products particularly in medicinal plants [8]. It has been

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documented that organic fertilizer such as manure can improve the plant productivity with rarely environmental concerns [9,10].

Organic fertilizers are widely used to improve the agricultural production [10,11]. Although the qualitative and quantitate improvement of EO, and physiological characteristics of different organic and chemical fertilizers in medicinal plants has been well documented [12-16]. There is little information regarding the effect of cattle manure on physiological and biochemical properties of medicinal and aromatic plants (MAPs). Recently, MAPs have widely applied for their high capacity in neutralizing the toxic free radicals [17].

The management of plant cultivation can influence plant growth and development. Plant density is a management cultivation practice influencing the plant growth with adjusting soil water and nutrients [18]. The planting pattern makes a particular situation for plants with changing the microclimate and soil features. According to the environmental, geographical and edaphic factors, the plants adopt their appropriate plant density to use the optimum nutrients and water [19]. In the recent years, the excessive use of chemical fertilizers in agricultural systems boosts environmental challenges and concerns globally. The use of organic fertilizers is the strategy to subside the undesirable effects of chemical materials and environmental stresses on plant production. Although cattle manure is a main alternative of chemical fertilizers in Iran, there is little knowledge about its effect on medicinal plants particularly *Satureja.* Therefore, we investigated the effects of organic fertilizers (cattle manure) and plant density on chlorophyll content, plant moisture, Malondialdehyde (MAD), phenol and flavonoid contents, and EO percentage of Bakhtiari savory under dryland farming condition.

Material and Methods

Site Description

Bakhtiari savory seeds were obtained from Research Institutes of Forests and Rangelands (RIFR), Iran. The seeds were sown in the trays filled with perlite and coco peat (1:1 volumes) at the greenhouse in RIFR. After 60 days, the seedlings were transplanted to the field in Damavand county (52º 20″ E and 35º 42″ N), Tehran province, in April 14th, 2018.

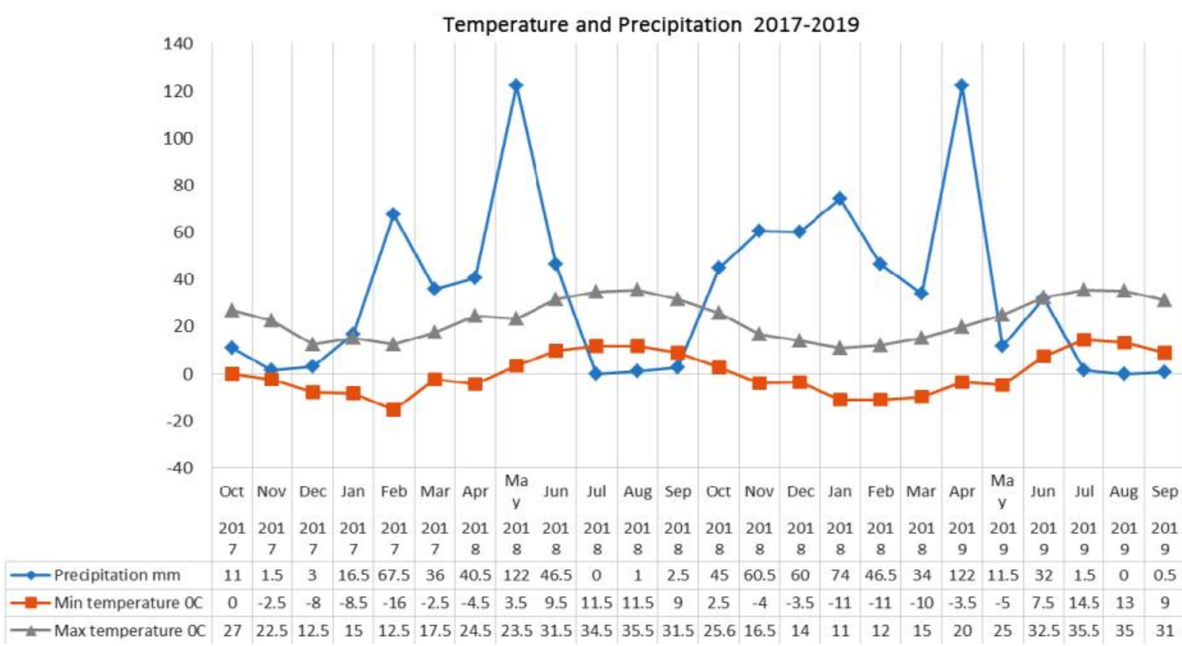

Fig. 1 The ambrothermic diagram during 2017- 2019

Table 1 Soil properties in two soil depth (0-20 cm and 20-40 cm)

Year	Depth	pH	EC	OC (%)	N%	P (mg/kg)	K (mg/kg)	Sand (%)	Silt (%)	Clay (%)
2018	0-20 cm	8.1	0.8	1.1	0.97	18	270	21	46	33
	20-40 cm	8.3	0.4	1.1	0.98	17	255	21	42	37
2019	0-20 cm	8.1	0.9	1.3	0.96	21	273	20	47	33
	20-40 cm	8.2	0.4	1.2	0.95	19	259	21	43	36

The mean annual temperature was 12 °C, which it's minimum and maximum occurred in January–February (-15 °C) and July–August (35 °C), respectively. Mean annual rainfall was 340 mm. Figure 1 represents the ambrothermic diagram during 2017 and 2019. The soil properties are presented in Table 1.

Experimental design and treatment details

A split plot experiment based on a randomized complete block design (RCBD) with three replications was conducted in 2018- 2019. Two levels of fertilizer application (0 and 30 ton ha^{-1} cattle manure) were assigned as the main plot and three levels of plant density (high plant density (HPD) as 80000 plant ha^{-1}, medium plant density (MPD) as 40000 plant ha^{-1}, and low plant density (LPD) as 26666 plant ha^{-1}) were used to the sub plots. The spacing between rows was 50 cm and within rows were 25, 50, and 75 cm for HPD, MPD, and LPD, respectively. Cattle manure as an organic fertilizer was mixed to the soil before transplanting. During the experiment, no pesticide and chemical fertilizers were used and weeds controlled manually. In both years, plants were harvested at flowering stage at the end of September.

Chlorophyll (Chl) assay

The contents of Chl a and b were extracted according to [20]. 200 mg of fresh samples were homogenized in 8 ml 80% acetone. After that, the mixture was centrifuged at 4 °C for 15 min (3000 rpm). Supernatants were used for analyzing chlorophyll content. Absorbance was determined at 645 and 663 nm by the spectrophotometer.

Relative water content (RWC) measurement

The RWC of leaves was calculated as a percentage according to the method of Dhopte and Manuel [21] as follows:

$$RWC = \frac{(FW - DW)}{(SW - DW)} \times 100$$

Where, FW is fresh weight, SW is leaf weight after soaking for 24 hours at room temperature and DW is leaf dry weight after drying for 24 h at 75 °C.

where, wf is fresh weight, ws is leaf weight after soaking for 24 hours at room temperature and wd is leaf dry weight after drying for 24 hours at 75 °C.

Malondialdehyde (MAD) concentration

To determine the MAD content, the samples were extracted with phosphate buffer and centrifuged at 14,000 rpm for 30 min. After that, the thiobarbituric acid (0.5% w/v) containing 20% w/v trichloroacetic aci was added to the mixture. The samples were placed in a hot water bath for 30 min and then were immediately cooled with ice and finally centrifuged at 10,000 rpm for 10 min. Samples were read at 532 and 600 nm wavelengths [22].

Proline Concentration

To measure proline content, 0.5 g fresh leaf sample was mixed with 10 ml of sulfosalicylic acid (3% w/v). The mixture containing the sample were centrifuged at 4000 × g for 20 min, and then 2 ml of ninhydrin acid and 2 ml of glacial acetic acid were added and vortexed. Simultaneously, 2 ml of standard 0, 4, 8, 12, 16, 20 mg l^{-1} proline and 2 ml ninhydrin acid and 2 ml acetic acid were mixed and vertexed. All samples were heated in a hot water bath for 60 min and then placed on ice to be cooled completely. 4 ml of toluene was added to the solution and stirred with Vertex for 20 sec. Using 0, 4, 8, 12, 16 and 20 mg l^{-1} proline standards, the standard curve was determined spectrophotometrically at 520 nm. The toluene soluble proline was sufficiently was measured at 520 nm expressed as µmol proline g^{-1} FW [23].

Determination of Total Phenolic Content (TPC)

Folin–Ciocalteu reagent was selected to measure TPC spectrophotometrically [24]. 100 µl of the MeOH solution of the precisely measured weight of investigated plant 1–10 (2.54, 2.58, 2.25, 4.03, 4.80, 2.13, 4.62, 1.47, 1.58, 15.05 mg mL^{-1} respectively) were mixed with 0.75 mL of Folin–Ciocalteu reagent and allowed to stay at 22º C for 5 min. The mixture was supplied with 0.75 ml of $NaHCO_3$. Absorbance was measured at 725 nm by UV–VIS spectrophotometer (Varian Cary 50) after 90 min at 22 ºC. Standard curve was calibrated by Gallic acid (0–100 mg ml^{-1}; $r > 0.99$). The results were represented as mg Gallic acid (GA) g^{-1} Dry weight.

Determination of Total Flavonoid Content (TFC)

The flavonoid levels were measured by aluminum chloride colorimetric method [25]. Briefly 0.5 ml of extract solution with 1.5 ml of 95% ethanol, 0.1 ml of aluminum chloride 10%, 0.1 ml of 1 M potassium acetate were mixed with 2.8 ml of distilled water. The mixture vortexed for 10 s and left to stand at 25 ºC for 30 min. The absorbance of the mixture was read at 415 nm. Quercetin concentrations (0 to 1200 µg ml^{-1}) were prepared and linear fit was used for calibration of the standard curve.

Essential oil (EO) Content

EO content of flowering branches was quantified using the method described by the European Pharmacopoeia for oil production [26]. Briefly, 100 g of dried aboveground plant parts were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus.

Statistical analysis

The data ($n = 3$) were subjected to one-way analysis of variance (ANOVA) and using the SAS software package for Windows (SAS, version 9.3, SAS Institute, Cary, NC). Duncan's multiple range tests showed the comparison of mean values. The data were statistically investigated at 5% probability level.

Results

Chlorophyll Content (Chl)

The concentration of chlorophyll (Chl a) was significantly influenced by manure application and plant density during two years (*P*≤0.05, Table 2). Chl a in the second year was higher compared to the first year. In plants treated with manure application and MPD, Chl a in the second-year plants increased by 26% compared to first-year plants (Table 2). Manure increased the Chl content particularly in the second year, and therefore we found a 20% enhancement of Chl a under manure application comparing to non-manure application at MPD in the second year. Chl b was affected by manure application (*P*≤0.05, Table 2), and increased by 19% at LPD with non-manure treatment in second year compared to first year (Table 2). According to the positive effect of manure, 12%-increased Chl b was achieved in first year with LPD in plants treated with manure relative to non-manure treatment (Table 2).

Relative Water Content (RWC)

RWC was affected by fertilizer and plant density (*P*≤0.05, Table 3). RWC ranged from 69% in first-year plants at LPD with non-manure application to 86% in in second-year plants at HPD supplied with cattle manure. In the second-year plants under HPD, we observed increased RWC (12%) compared with control plants (Table 3).

Malondialdehyde (MAD) and Proline Concentration

The significant changes in MAD were recorded by fertilizer and plant density (*P*≤0.05, Table 3). The highest MAD (4.5 µ mol g^{-1} FW) was observed in plants at LPD with non-manure application (table 3). Proline as an essential component of plant cells was influenced by plant density and organic fertilizer (*P*≤0.05, Table 3). It increased by decreasing the plant density, where its highest concentration was obtained at LPD in the first year (Table 3).

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Organic fertilizer and plant density significantly affected the TPC of Bakhtiari savory during two growing seasons. TPC in the second year was significantly greater than the first year. At MPD with manure application, TPC significantly increased by 22% in the second year in comparison to the first year (Fig. 2). Organic fertilizer improved TPC content.

Table 2 Chlorophyll content of Bakhtiari savory under organic fertilizer and plant density

Year	Organic Fertilizer	Plant density	Chl.a (mg g^{-1} FW)	Chl.b (mg g^{-1} FW)	Chl.a/b
2018	Control	Low	0.74±0.017 e	0.26±0.008 d	2.86±0.09 a
		Medium	0.74±0.008 e	0.29±0.024 b-d	2.60±0.20 a
		High	0.78±0.012 c-e	0.28±0.024 cd	2.73±0.21 a
	Manure	Low	0.78±0.012 c-e	0.28±0.020 cd	2.66±0.20 a
		Medium	0.76±0.013 de	0.31±0.029 a-c	2.49±0.28 a
		High	0.80±0.012 cd	0.33±0.012 a-c	2.46±0.12 a
2019	Control	Low	0.80±0.017 cd	0.31±0.012 a-c	2.60±0.09 a
		Medium	0.80±0.022 cd	0.32±0.013 a-c	2.48±0.13 a
		High	0.81±0.008 c	0.33±0.008 a-c	2.45±0.08 a
	Manure	Low	0.90±0.017 b	0.34±0.017 a	2.62±0.11 a
		Medium	0.96±0.009 a	0.34±0.016 a	2.84±0.14 a
		High	0.94±0.012 a	0.34±0.011 a	2.81±0.14 a
Significance	Year (Y)		**	**	ns
	Fertilizer (F)		**	**	ns
	Y*F		**	ns	**
	Density (D)		**	ns	ns
	Y*D		*	ns	ns
	F*D		ns	ns	ns
	Y*F*D		ns	ns	ns

ns: non-significant; *: Significant at p≤0.05; **: Significant at p≤0.01

Table 3 Relative water content (RWC), Malondialdehyde (MAD) and proline concentration of Bakhtiari savory under organic fertilizer and plant density

Year	Organic Fertilizer	Plant density	RWC (%)	MAD (µ mol g^{-1} FW)	Proline (µ mol g^{-1} FW)
2018	Control	Low	69.0±2.1f	4.5±0.21 a	233.7±2.8 a
		Medium	71.3±1.7 ef	3.9±0.12 b	220.7±1.7 b
		High	75.7±1.6 c-e	3.4±0.16 c	223.3±2.6 b
	Manure	Low	77.3±2.4 b-d	4.2±0.12 ab	200.0±1.6 c
		Medium	81.0±2.1 b	3.2±0.14 cd	190.7±4.9 de
		High	86.0±0.8 a	3.1±0.08 cd	185.0±1.6 e
2019	Control	Low	73.0±1.7 d-f	4.0±0.17 b	195.0±3.5 cd
		Medium	75.3±2.1 c-e	3.2±0.13 cd	163.7±2.8 fg
		High	80.3±1.2 bc	2.9±0.09 d	168.3±1.2 f
	Manure	Low	77.3±1.7 b-d	3.4±0.21 c	160.7±4.9 gh
		Medium	86.0±2.9 a	3.4±0.12 c	156.3±4.1h
		High	89.0±2.4 a	3.3±0.12 c	158.0±1.7 gh
Significance	Year (Y)		**	*	**
	Fertilizer (F)		**	**	**
	Y*F		ns	**	*
	Density (D)		**	**	*
	Y*D		ns	*	ns
	F*D		ns	*	**
	Y*F*D		ns	**	**

ns: non-significant; *: Significant at p≤0.05; **: Significant at p≤0.01

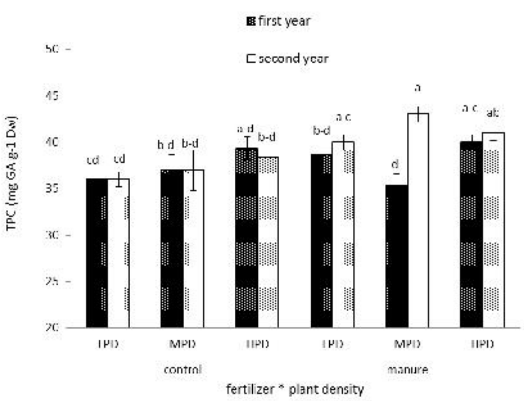

Fig. 2 Total phenolic content (TPC) under organic fertilizer and plant density during 2018 and 2019

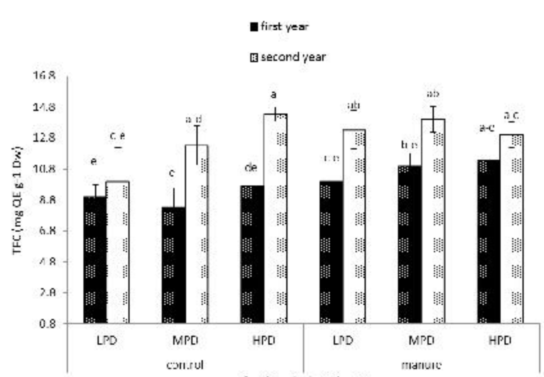

Fig. 3 Total flavonoid content (TFC) under organic fertilizer and plant density during 2018 and 2019

For example, at MPD in the second year, we observed 17% increased TPC with manure application compared to its no use (Fig. 2). The effect of organic fertilizer was significant on TFC. The use of cattle manure improved the TFC. In the first year at HPD, manure application increased TPC by 21% compared to the non- manure treatment (Fig. 3).

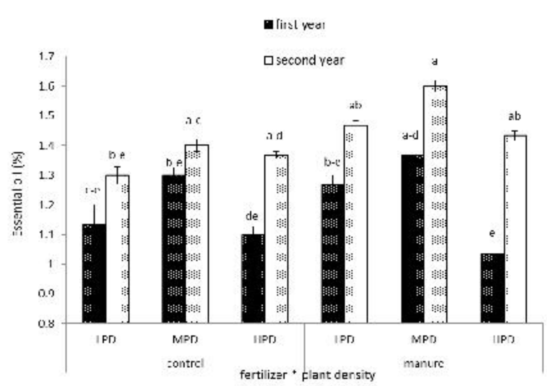

Fig. 4 Essential oil (EO) content under organic fertilizer and plant density during 2018 and 2019

Essential oil (EO) Percentage

EO content was significantly affected organic fertilizer and plant density. To obtain the highest EO content, the plants should be harvested in the second year. EO percentage in MPD was higher than other plant densities. The highest EO percentage (1.6%) was observed at MPD with manure application in the second year (Fig. 4).

The Correlation between Studied Traits

The correlation of the studied traits is described in Table 4. Chl a was positively correlated with chl b (r=0.619), RWC (r=0.650), TPC (r=627), TFC (r=0.631), and EO (r=0.555), but was negatively correlated with MAD (r=-397) and proline (r=-0.784). We observed a significant and positive correlation between RWC and TPC (r=0.511), TFC (r=540), but a significant and negative correlation was obtained between RWC and MAD (r=-622) and proline (r=-652). The close correlation was also obtained for proline content with TPC (r=-435), TFC (r=-808), and EO (r=-601). TFC was significantly correlated with EO content (r=484).

Discussion

The optimum Chl contents were determined in plants supplied with cattle manure in the second year. The essential micro and macro-nutrients in manure such as nitrogen, phosphorus, potassium, and zinc promote plant growth. Manure can fertilize the soil through increasing the organic matter and improving the physicochemical properties of soil such as soil structure, aeration, moisture, and pH [27]. Moreover, manure due to its potential plays a significant role in chlorophyll contents by supplying the fraction and improving the solubility of the main elements [4,28]. The positive effects of organic fertilizer on chlorophyll concentration also have been reported in other plants [29]. We found increased Chl content in the second year relative to the first year. Plants are capable to adapt the new environmental condition by time; thereby in second year have more potential in nutrients uptake, resulting the improved Chl concentration. In addition, it could be noticed that the decomposition of manure happens at second year, resulting more nutrients viability for plants. In high and MPD, the increased moisture of soil improves root condition in nutrient and water uptake to get the highest photosynthesis rate. Since the present study was carried out in a dryland farming condition, the dense canopy can maintain the soil moisture. Sufficient soil moisture provides the uptake of plant nutrients, which subsequently increase the plant growth. Saki *et al*. [4] reported an increase growth of *Satureja mutica* Fisch. & C.A.Mey. due to optimum photosynthesis rate in HPD. In LPD, we observed the reduced Chl concentration because of lower leaf area and later canopy development. Under dryland farming condition, the open canopy could not control sunlight rate, and it makes adverse effects on soil properties such as texture, moisture, and nutrients. RWC decreased at LPD, but increased at HPD and MPD. The reduction of RWC at MPD is due to decreased soil moisture induced by high light rate. In dryland farming condition, the open canopy allows adverse effects of sunlight rate on soil properties such as texture, moisture, and nutrients [30]. Manure due to its high potential in improving nutrients uptake and water capacity has a significant role on plant water content. Decreased RWC results in the decline of photosynthesis and plant growth, in which the close and positive correlation between RWC and Chl content in our study approved this phenomenon (Table 4).

The increased content of MAD indicates that the lack of moisture by producing oxygen free radicals can lead to oxidation of cell membrane lipids [31]. Increased MAD content under conditions of moisture deficiency have also been reported in other studies [32]. In his regard, the negatively close correlation between RWC and MAD supports the increasing trend of MAD with reducing RWC (Table 4). The researchers concluded cumulative proline in plant cells is a response to osmotic stress in a variety of plant species [33]. In our study, at LPD, proline increased due to low water content in soil.

The most common pathway for proline synthesis in plants is the glutamate pathway and during the water stress more glutamate is converted to proline.

Table 4 The correlation between the studied traits

	Chl a	Chl b	RWC	MAD	Proline	TPC	TFC	EO
Chl a	1	-	-	-	-	-	-	-
Chl b	0.619**	1	-	-	-	-	-	-
RWC	0.650**	0.536**	1	-	-	-	-	-
MAD	-0397*	-0.664**	-0.622**	1		-	-	-
Proline	-0.784**	-0.785**	-0.652**	0.654**	1	-	-	-
TPC	0.627**	0.307	0.511**	-0.271	-0.435**	1	-	-
TFC	0.631**	0.623**	0.540**	-.624**	-0.808**	0.420*	1	-
EO	0.555**	0.489**	0.242	-0.267	-0.601**	0.247	0.484**	1

*: Significant at p≤0.05; **: Significant at p≤0.01. EO: Essential oil. RWC: Relative water content, MAD: Malondialdehyde, TPC: Total phenol content, TFC: Total flavonoid content.

Decreased proline degradation, degradation, and disruption of the protein synthesis process also play a role in increasing proline in low water potential [33]. At LPD, the intensity of sunlight is high and evaporates the soil water easily and therefore makes a stress in plants. Proline stabilizes the membranes and macromolecules and helps maintain their natural shape and structure under low levels of soil moisture. In addition to its direct effect on stabilizing macromolecules, proline also has an indirect protective effect due to its antioxidant properties [34]. Antioxidant capacity increased over time, with manure application and increased plant density. Flavonoids and phenolics contents increased with manure application. Organic fertilizers with affecting the acetate shikimate pathway improve phenolic content of medicinal plants [4]. Additionally, because of the higher photo–pathogenic stress in organic farming, this in turn might induce abiotic stress, and increase phenolic compounds [35]. Organic fertilizers increased antioxidant potential of *Satureja multica* [4, 35-37]. Antioxidant capacity of second year was higher in compare to first year. TPC and TFC of plants could be raised by time due to the change in physiological responses of plants to the duration that plant is exposed the ecological and edaphic parameters such as temperature, light, and soil acidity [35]. Increased antioxidant activity was observed with medium and HPD. The increased competition of plants to obtain sufficient light, nutrients, and water is associated with HPD, which results in raising the antioxidant activities such as TPC. The increased TPC due to HPD was observed by Taleie *et al*. [38] in *Stevia rebaudiana* (Bertoni) Bertoni, Lombardo *et al*. [39] in *globe artichokes*, Danesi *et al.* [40] in palm tree kale, and by Saki *et al.* [4] in *Satureja mutica.* Increased EO content was observed in plants supplied with the interaction of manure application and second year compared to no application of manure and also first year. Previous studies have revealed that organic fertilizers improved EO quantity and quality of some medicinal plants [4, 10, 41-44]. Manure upgrades the soil characteristics along with increasing the benefit microorganism in rhizosphere. It improves the plant growth, nutrients uptake, and stimulates physiological and biochemical pathways of plants such as EO production [45]. In the present study, we observed an increased EO yield at MPD. EO production is influenced by the interaction of various factors like soil texture, photosynthesis rate, temperature, precipitation etc. In general, the factors affecting photosynthesis can promote the EO production. We determined the positive correlation between photosynthesis content and EO concentration. The photosynthesis and EO production significantly were affected by plant density through changing the canopy, which finally alters the sunlight rate, soil moisture, canopy temperature, soil nutrients [46].

Conclusions

The present study attempts to find the best plant density on some physiological and biochemical properties of Bakhtiari savory with manure application under dryland farming. Plant density is an eminent factor in determining the quality and quantity of plants. In addition, organic fertilizers due to its environmental-friendly and also human health features are strongly capable to be used as an alternative of chemical fertilizers. Therefore, we evaluated the effect of plant density and cattle manure on physiological properties and essential oil of Bakhtiari savory. It was concluded that in the semiarid areas like most parts of Iran (with about mean annual rainfall of 340mm) under dryland farming, we can archive the optimum antioxidant capacity and essential oil quantity of Bakhtiari savory at MPD or HPD with manure application in the second year. The improved plant production in the second year compared to first year could be due to the fact that cattle manure is decomposed and released its nutritional values in the soil slowly.

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Conflict of Interest

There was no conflict of interest between the authors.

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