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Effect of Plant Density and Nitrogen Rate on Essential Oils of Marjoram under Mediterranean Conditions

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Abstract. Quantitative analysis of essential oils of marjoram, which is grown in Jordan, yielded thymol as major compound. The yield of essential oils increased with the reduction of plant density. The increase in nitrogen dose (from 50 to 100 kg/ha) correlated with the increase in the yield of essential oils in all harvests, while with the increase in the dose of nitrogen from 100- 150 kg/ha, the yield of essential oils decreased. On the other hand, the nitrogen dose of 150 kg/ha correlated with essential oils containing the highest percentage of thymol (53.11%). A strong relation was found between the yield and the chemical composition of marjoram essential oils and applied agronomic treatments which require special attention.

Keywords: essential oils, *Origanum syriacum*, nitrogen dose, plant density

Introduction

Marjoram (*Origanum syriacum*) contains essential oils, which have a number of nutraceutical properties, including anti-microbial and antioxidant properties (Deans and Ritchie, 1987; Conner and Beuchat , 1984). Lawrence and Reynolds (1994) reported that chemical composition and the concentration of individual essential oils are variable. They further found that the concentrations of two predominant compounds of thyme essential oils, i.e. thymol and carvacrol, ranged from as low as 3% to as high as 60% of the total essential oils. Essential oils are reported to have fungicidal properties in pharmaceutical, cosmetic and perfumery industries (Manou *et al.,* 1998; Lawrence and Reynolds, 1994). Many studies have demonstrated the importance of phenolic compounds in plant defence response (Appel, 1993; Harborne, 1991). There are many indications that the biosynthesis and metabolism of terpenes in aromatic plants are influenced by environmental factors. Early studies have shown that environmental and management factors such as temperature, water stress, soil fertility, light, pest pressure, cutting date and plant maturity are important factors affecting concentration of phenolic compounds in plants (Ihsan *et al.,* 1999; Mairapetyan *et al.*, 1999; Omer, 1999; Hassan *et al.*, 1997; Letchamo and Gosselin, 1996; Li *et al.*, 1996; Randhawa and Kaur, 1996; Takano and Yamamoto, 1996; Zheljazkov and Margina, 1996; Grahle and Holtzel, 1993; Piccaglia *et al.,* 1993; Takano, 1993). Although effects of environmental factors on marjoram essential oils have been well documented and researched, information on the effect of specific agronomic practices remains limited. The objective of this study was to determine the main effects and interactions of plant density and nitrogen level on the yield and chemical composition of essential oils of marjoram.

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Materials and Methods

Site description. Field trials were carried out at the field site of Jordan University of Science and Technology (JUST) campus in northern Jordan [32° 34' N latitude, 36° 01' E longitude, 520 m altitude] during the two growing seasons of 2000-01 and 2001-02. The location has Mediterranean climate of mild rainy winter and dry hot summer. The soil is fine loamy, thermic, calcic paleargid. Important characteristics of the soil (0-15 cm depth) are summarized in Table 1.

Land preparation and crop culture. Experimental area was subjected to two perpendicular chisel plow passes during the fall season. Field was disked before transplantation to provide a proper seedbed and experimental plots of 2.25 × 1.8 m were established. The experiments were arranged in a randomized complete block design (RCBD) and replicated three times. Plowing operations were performed by chisel to a depth of 20 to 25 cm followed by rotary harrow to level soil surface. Four month old marjoram seedlings, with an average height of 20 cm, were obtained from Nour Al-Hussein Foundation (NAHF). Hardening of seedlings before transplanting was accomplished by exposing them to low temperature (8 °C), so

Table 1. Pre-experimental physical and chemical properties of the study soil (top 15 cm layer)

Soil characteristics	Value
$CaCO_3$ (mg/kg)	13.0
Organic matter (%)	0.79
N-NH_4 (mg/kg)	5.25
N-NO_3 (mg/kg)	16.78
Available P (mg/kg)	3.33
pH	7.93
Sand (%)	5.5
Silt (%)	61.0
Clay (%)	33.5

as to let them adapt to the JUST field conditions during winter season. Transplanting was carried out to the experimental plots in May, 2001. Each plot consisted of four rows; the outer ones were considered border line. Seedlings were drip irrigated, at intervals and the amount of water was adjusted to maintain optimum moisture availability; total amount of water provided during the whole growing season was 800 m^3/ha. Three cuttings were done during the first growing season (2001), in July, August and October. During the second season (2002), three further cuttings were done in May, July and September. Cutting height was around 7cm above soil surface.

Treatments. The treatments were factorial combinations of four plant densities (4, 6, 8 and 14 plants/m^2) and four rates of application of nitrogen fertilizer (0, 50, 100 and 150 kg N/ha). Plant densities of 4, 6, 8 and 14 plants/m^2 were maintained by keeping distances of 60, 45, 30 and 15 cm between plants of the same row, while distance between rows was kept constant at 75 cm for all treatments. Nitrogen fertilizer was added in the form of urea $(NH_2)_2Co$ (46% N) at transplantation and a day after each harvest, all of which resulted in total application rate of 0, 50, 100 and 150 kg/ha per season. Nitrogen fertilizer treatments varied according to the rate of application and were devised to provide for control (with no fertilizer) and various rates of split application.

Essential oil distillation, Marjoram plants *(Origanum syriacum* L.) were collected from the field after each harvest. The plants were dried in oven at room temperature for some days. Dehydrated plants were weighed and powdered in blender, then macerated for 24 h in 90 ml distilled water at room temperature. Steam distillation was performed for 4 h by modified steam distillation apparatus. The essential oil was extracted from the distillate by chloroform ($CHCl_3$). Chloroform layer was separated and then dried with Na_2SO_4. Chloroform was evaporated under reduced pressure (*in vacuo*) at 35 °C by using rotary evaporator. The essential oil content was determined gravimetrically on shoot dry matter basis. Quantity of the extracted oil from plant leaves was determined on fresh weight basis.

Qualitative and quantitative analysis of oils. Thin layer chromatography (TLC) was performed as cited by Wagner *et al.*, (1984) for the qualitative identification of thyme oil, on silica gel plates (stationary phase). The mobile phase used in TLC was 93% tolouene + 7% ethyl acetate. Reagents were ethanolic (conc.) sulphuric acid 95:5 (solution I) and ethanolic vanilin 99:1 (solution II). Detection was performed by spraying vigorously the silica gel plate with solution I (10 ml) and immediately afterwards with solution II (5-10 ml). Silica gel plates were oven dried at 110 ºC for one min. The quantitative determination of different oil samples was done by using GC (Varian 3300 Gas Chromatograph) equipped with flame ionization detector (FID), coupled to an integrator (Unicam). For separation of thymol, column of stainless steel (2 m x 1/8") was packed with 15% Carbo Wax 20 M, with W 80-100 mesh. The carrier gas was N_2 with flow rate of 16 ml/sec. The injector and detector temperatures were held at 240 °C and 250 °C, respectively. The essential oil samples were diluted with *n*-hexane (HPLC grade), 4 μl were injected from samples of plant tissue oil and 2μl were injected from samples of plants. Oil samples of each treatment were analyzed separately.

Statistical analysis. The data was analyzed as RCBD (randomized complete block design) with two factors, plant density and N fertilizer rate. The means were separated by using Fisher's least significant differences (LSD) according to Steel and Torrie (1980) by using MSTAT-C software (Michigan State University, 1988).

Results and Discussion

The percentage of volatile oil in the air dried herb gradually increased with the increase in plant age. Thereafter, a decline occurred towards the end of the growing season. Differences in oil content among the harvest dates can probably be attributed to differences in various factors including photosynthesis, temperature and light levels. Light levels and temperature were specially high during growth period of the plants (Table 2) which contained the highest oil percentage i.e. 1.84% at the 2nd harvest, compared to 1.64% at the 1st harvest and 1.67% at the 3rd harvest. Biosynthesis of the secondary metabolites has been related to the capture of light energy. In general, light stimulates the synthesis of phenolic

Table 2. Maximum and minimum air temperature during 2001-02

Month	Maximum (°C)		Minimum (°C)	
	2001	2002	2001	2002
January	15.48	11.76	4.45	3.61
February	15.26	16.24	5.17	6.19
March	23.51	21.27	9.49	7.59
April	25.28	21.75	11.96	8.99
May	26.21	27.35	13.98	11.45
June	32.88	31.46	16.61	15.58
July	33.97	34.06	18.15	19.29
August	33.66	33.28	18.42	18.28
September	30.78	32.06	16.42	16.28
October	28.14	29.44	14.23	16.11
November	20.08	22.29	8.41	9.53
December	16.06	14.78	6.60	5.74

compounds. Various researchers have reported an increased production of phenolic compounds with UV radiation stress (Caldwell *et al.*, 2005; Kubasek *et al.*, 1992; Tevini *et al.*, 1991; Schmelzer *et al.*, 1988). It has been reported that plant flavonoid biosynthesis genes are transcriptionally activated by light, whereas phenolic compounds may provide defence against ultraviolet light (Stapleton, 1992). Hughes *et al.* (1999) found that exposure of root systems of alder plants to light can promote the synthesis of flavonoids. They also observed increased levels of phenolic compounds in plants supplemented with UV light.

There was no interaction between the plant density and the nitrogen rate; results are therefore presented for main effects only. Highly significant differences ($P \leq 0.01$) in essential oil percentage values were recorded between the plant density treatments (Table 3). The present study as well as the literature suggest that the essential oil percentage significantly increases with decrease in plant density (Ihsan *et al.,* 1999). The increase in number of essential oil glands in the leaves of the plants, grown under supplemental light, compared to those, grown under natural light, is in agreement with the findings of Yamaura *et al.* (1989); he indicated that *Thymus vulgaris* seedlings, which developed under light, produced more glandular trichomes than those, developed in darkness. The essential oil glands are secretions from capitate or peltate glandular trichomes, and may contain both lipophilic and polysaccharide substances in other examined species of the Labiatae. The positive correlation between the number of essential oil glands and oil content may suggest that the

Table 3. Effect of plant density and rate of nitrogen fertilizer application on essential oil yield of marjoram during 2001-02

N fertilizer (kg/ha)	PD (plants/m^2) 14	8	6	4	Mean
	Essential oil (%)				
First harvest					
0	1.25	1.43	1.64	1.72	1.51
50	1.53	1.52	1.69	1.82	1.64
100	1.79	1.62	1.82	1.91	1.79
150	1.54	1.64	1.63	1.72	1.63
Mean	1.53	1.55	1.70	1.79	LSD=0.08
Second harvest					
0	1.62	1.63	1.74	1.82	1.70
50	1.72	1.83	1.83	1.93	1.83
100	1.91	1.92	2.01	2.13	1.99
150	1.72	1.84	1.93	1.92	1.85
Mean	1.74	1.80	1.88	1.95	LSD=0.06
Third harvest					
0	1.43	1.52	1.64	1.75	1.59
50	1.53	1.61	1.73	1.85	1.68
100	1.61	1.71	1.85	1.92	1.77
150	1.54	1.65	1.62	1.72	1.63
Mean	1.53	1.64	1.71	1.81	LSD=0.04

PD = plant density; LSD = least significant difference

concomitant increase in light may increase the formation of essential oil glands with subsequent increase in essential oil content. On the other hand, the lower content of essential oils of closely planted crop could be the result of mutual shading; there was more shedding of the older and lower leaves rich in volatile oil contents, thereby decreasing their proportion in the herb and ultimately causing the reduction in the content of essential oil (Randhawa and Kaur, 1996). This conclusion is somewhat consistent with the observations of Li *et al.* (1996), who reported that herb yield and essential oil production were directly related to the biomass production and thus to photosynthesis because both the herb yield and the essential oil production increased with increase in the light level. Peltate hair are important site for the synthesis and the accumulation of essential oils in herbs, especially for the accumulation of monoterpenes, sesquiterpenes and diterpenes. The density of peltate hair of marjoram significantly increased as light level increased, with the highest derivative occurring at 100% sunlight. In the first harvest, the density of 4 plants/m^2 yielded significantly higher amount of essential oils compared to the densities of 6, 8 and 14 plants/m^2 and differences were 5.02%, 13.41% and 14.52%, respectively; densities of 8 plants/m^2 and 14 plants/m^2 had no significant difference. Similar trend was obtained at the second harvest, at which density of 4 plants/m^2 gave significantly higher yield of the essential oils (1.95%) than the other treatments. This increase was 3.59% over the densities of 6 plants/m^2, 7.69% over 8 plants/m^2 and 10.77% over 14 plants/m^2. The lowest plant density (4 plants/m^2) gave the highest mean essential oil yield (1.81%) which was higher than 6, 8 and 14 plants /m^2 densities by 5.52%, 9.39% and 15.47%, respectively, at the third cutting (Table 3). Highly significant differences ($P \leq 0.05$) in essential oil percentage were noted between different nitrogen rate treatments. Application of nitrogen increased the oil content by 15.6%, 14.6% and 10.2% in the first, the second and the third harvest of the second season, respectively (Table 3). Increasing nitrogen application from 50 to 100 kg/ha increased the oil content in all harvests which then decreased with the increase in nitrogen application upto 150 kg/ha, but was still higher than the level of oil in control plants. It means that essential oil content gradually declined with the increase in nitrogen rate over 100 kg/ha. The response of volatile oil content to nitrogen application might be attributed to the increase in the capacity of *de novo* meristematic cell metabolism in building dry matter and/ or its compensation with production of active ingredients with the result that the plant demands higher amounts of nutrients such as nitrogen (Omer, 1999). In this respect, Guenther (1973) claimed that the precursor of essential oil could be obtained through degradation of carbohydrates and proteins. Pridham (1976) also mentioned that certain compounds, like sugar and amino acids, can be translocated into the site of essential oil synthesis and are ultimately used during synthesis of monoterpenes. In that sense, it seems that the increase in nitrogen application leads to realizing a case of steady state or absence of competition on carbon skeletons necessary to meristematic and volatile oil production through acetate while nitrogen thresholds had stabilized the maintenance of nitrogen necessary for the growth and the activity, e.g. of proteinaceous enzymes.

It was observed that the oil concentration decreased with increasing nitrogen application to 150 kg/ha, being greater than that of control; it was probably because plants growing with nitrogen fertilization were less mature and were at earlier stage of development than those without nitrogen.

Results of the first harvest in the second season showed that at the application rate of 100 kg N/ha, the highest essential oil yield was obtained (1.79%), which was higher than nitrogen application of 150, 50 and 0.0 kg/ha, by 8.94%, 8.38% and 15.64%, respectively. Application of 100 kg N/ha gave yield of essential oil higher than that of 150 kg, 50 kg and 0 kg N/ha by 7.03%, 8.04% and 14.57%, respectively, at the second harvest in the second growing season. Similar trend was observed at the third harvest (second growing season), at which application of 100 kg N/ha gave significant yield (1.77%) compared to those of other treatments. This increase was 7.91% over application of 150 kg N/ha, 5.08% over that of 50 kg N/ha and 10.17% over the control.

The main constituents of essential oil of marjoram plants are thymol and its isomer carvacrol, followed by their hydrocarbon precursors (*p*-cymene and ã-terpinene). There was a good correlation between the fresh thymol yield of marjoram and the three harvest dates. Thymol percentage at the second harvest (57.18%) was much higher than that of the first harvest (50.14%) by 12.31%, and was higher than that of the third harvest (39.74%) by 30.94%. The increase in thymol percentage after the first harvest was probably due to increase in the temperature in warm season and hence decrease in thymol at low temperature after the second harvest. Similar results were reported by Omer (1999), who found that the thymol and carvacrol were the two main constituents of oregano oil in three successive cuttings from mid-summer to early winter, followed by their hydrocarbon precursors (*p*-cymene and ã-terpinene). Main differences were recorded with these four compounds in which phenolic terpene compounds, thymol and carvacrol, increased in warm season at the expense of their preceding precursors. Higher temperatures were found favourable for accumulation of higher thymol contents. Thymol percentage was significantly ($P \leq 0.05$) affected by plant density (Table 4). The general trend indicated that thymol yield increased significantly with decreasing plant density. The lowest density (4 plants/m^2) gave the highest yield for all harvests (53.39 %). Similar results have been obtained with *Mentha arvensis* L.(Randhawa and Kaur, 1996), sage *(Salvia officinalis*) and thyme (*Thymus vulgaris*) (Li *et al.*, 1996). Increase in thymol percentage of *Origanum syriacum* with decrease in plant density was probably due to the solar radiation available around the plants. At the first harvest, plant density of 4 plants/m^2 gave significantly higher yield compared to the densities of 6, 8 and 14 plants/m^2, the difference being 5.19%, 11.85% and 20.51%, respectively. Similar trend was observed at the second harvest, at which density of 4 plants/m^2 gave significantly higher yield (60.91%) than other densities. This increase was 3.05% over 6 plants/m^2, 8.13% over 8 plants/m^2 and 12.83% over 14 plants/m^2. The lowest density (4 plants/m^2) gave the highest mean yield (43.91%) which was higher than that of 6, 8 and 14 by 7.01%, 12.43% and 18.58%, respectively, at the

Table 4. Effect of plant density and rate of nitrogen fertilizer application on thymol yield of marjoram during 2001-02

N fertilizer (kg/ha)	PD (plants/m^2) 14	8	6	4	Mean
	Thymol (%)				
First harvest					
0	39.05	44.33	48.47	52.36	46.05
50	40.35	47.86	51.21	53.45	48.22
100	46.33	49.65	52.65	56.01	51.16
150	50.21	53.26	57.56	59.54	55.14
Mean	43.99	48.78	52.47	55.34	LSD=1.65
Second harvest					
0	49.25	52.36	56.65	57.69	53.99
50	51.24	55.32	58.22	59.26	56.01
100	54.32	56.48	60.01	62.35	58.29
150	57.55	59.68	61.33	64.32	60.72
Mean	53.09	55.96	59.05	60.91	LSD=2.09
Third harvest					
0	32.25	35.35	39.36	41.65	37.15
50	34.26	36.54	38.54	42.01	37.84
100	37.45	39.56	40.32	44.65	40.49
150	39.04	42.34	45.13	47.35	43.47
Mean	35.75	38.45	40.83	43.91	LSD=2.35

PD = plant density; LSD = least significant difference

third harvest. The analysis of variance showed that there were highly significant ($P \leq 0.01$) effects of nitrogen fertilizer application rate on thymol yield of marjoram plants at all harvests (Table 4). The highest thymol percentage (53.11 %) was recorded at the highest nitrogen application rate (150 kg N/ha); as the nitrogen application rate was increased, the thymol yield also increased (Table 4). However at the first harvest, 150 kg N/ha produced the highest yield of 55.14 % which was significantly higher than that of the application of 100 kg N/ha by 7.22%, 50 kg N/ha by 12.55% and 0 kg N/ha by 16.49%. At the second harvest, the highest nitrogen rate (150 kg N/ha) gave the highest mean fresh thymol yield of all the other plant densities (60.72%). The increase was 4.00% over 100 kg N/ha, 7.76% over 50 kg N/ha and 11.08% over 0 kg N/ha. With regard to the third harvest, the highest nitrogen rate (150 kg N/ha) gave the highest fresh thymol yield (43.47%) which was significantly higher than100 kg N/ha by 6.86 %, 50 kg N/ha by 12.95% and 0 kg N/ha by 14.54%. No significant differences were found when 100 and 150 kg N/ha were used. It is generally said that plants respond to sub-optimal soil fertility levels by increasing the synthesis and accumulation of phenolic compounds in their tissues. However, results of the studies investigating the effects of fertilization on phenolic compound concentration are contradictory; response may vary with species, elements and level of the stress. Seguin *et al.* (2003) found that K, P, S and B fertilization had limited impact on soybean seed flavonoid content in fields with medium to high soil fertility. On the other hand, Vyn *et al.* (2002) observed positive effects of K fertilization on flavonoid concentration of soybean seed on low to medium testing K soils. Carpena *et al.* (1982) reported that tomato response may vary depending on the elements. They reported that while B deficiencies resulted in increased flavonoid accumulation in leaves, P and Mn deficiencies did not affect the total flavonoid content but rather affected the types of flavonoids present. Stout *et al.* (1998) reported that, in greenhouse trials, tomato leaf grown at low nitrogen availability had greater flavonoid content. Most studies on the effects of mineral nutrition have been conducted under controlled conditions and there is still limited information on the impact of fertilizer application strategies on the concentration and the yield of phenolic compunds in field-grown marjoram under Mediterranean conditions.

Conclusion

The present study suggests that environmental and agronomic factors have a great impact on essential oil of marjoram (*Origanum syriacum* L) under Mediterranean conditions. The highest thymol yield and essential oil percentage were recorded at the lower plant density and the lowest yield at higher plant density. With the increase in application of nitrogen from 50 to 100 kg/ha, the oil content in all harvests increased but then tended to decrease with the increase in nitrogen application to 150 kg/ha. In contrast, the highest thymol percentage (53.11%) was obtained from the highest rate of nitrogen application (150 kg N/ha). It thus seems that certain agronomic practices may need to be tailored specifically for essential oil production if concentrations of essential oil in marjoram are to be maximized.

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