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Effect of Nitrogen Concentration of the Nutrient Solution on the Volatile Constituents of Leaves of *Salvia fruticosa* Mill. in Solution Culture

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Essential oils from hydroponically cultivated *Salvia fruticosa* were analyzed by GC-MS techniques. Three different levels of nitrogen (100, 150, and 200 mg/L) were used in the nutrient solution for the cultivation, using the nutrient film technique. A total of 79 compounds were identified, and qualitative and quantitative differences have been observed between the samples collected at full bloom (flowering stage) and at the end of the seed formation stage. 1,8-Cineole, β-caryophyllene, and viridiflorol were the predominant constituents in most cases. 13-*epi*-Manool was identified by using GC parameters and mass spectrum fragmentation pattern, whereas labd-7,13-dien-15-ol, a labdane type diterpene, was identified for the first time in the genus *Salvia*, using GC parameters and an authentic sample. The results obtained from GC-MS analyses were submitted to chemometric analysis.

KEYWORDS: ***Salvia fruticosa*** **essential oils; hydroponic cultivation; GC-MS; chemometrics**

INTRODUCTION

Salvia fruticosa Mill. (Labiateae), which is a common aromatic species in most countries of the Eastern Mediterranean Basin, has been largely investigated mainly for the content and composition of its essential oil (*1*−*4*). Information concerning the hydroponic cultivation of *Salvia* species is absent, and knowledge of the effect of mineral nutrition on their growth, essential oil content, and composition of aromatic and medicinal plants is generally limited. However, Davtyan (*5*) reported the essential oil of the substrate cultivation of aromatic and medicinal plants. The same author reported results concerning increases of the essential oil content under hydroponic cultivation conditions in the case of *Pelargonium roseum* Willd. and *Ocimum basilicum* L. (*6*). Recently, we have published two papers (*6*, *7*) concerning the effect of nitrogen and phosphorus on the yield and chemical composition of hydroponically cultivated *Origanum dictamnus* by using three different concentrations of these elements.

Our research on the soilless cultivation of medicinal and aromatic plants from Greece has as a main concern to explore the possibility to improve their yield by applying the appropriate cultivation techniques, especially those referring to plant nutrition, as well as the quality of their essential oils by using hydroponics as a tool for plant nutrition studies. However, the aim of the present study was to investigate the effect of nitrogen on the content and composition of the essential oils of *S. fruticosa* Mill. using the nutrient film technique (NFT) in two different growth stages, that is at the flowering stage and at the end of seed formation. The nutrient film technique, a solution culture method, commonly known as NFT, is a method of cultivation in which plants have their roots in a shallow stream of recirculating nutrient solution (*8*). The nutrient solution required for plant growth is discharged into the upper (inlet) end of a sloping watertight channel containing the plants and flows as a shallow stream (film) through the root system. At the lower end of the channel (outlet), the solution is collected in a catchment tank and pumped back to the inlet for recirculation.

The NFT, widely used for commercial vegetable production, is an excellent method for plant nutrition studies (*9*), and most research has been directed toward them.

MATERIALS AND METHODS

Chemometric Statistical Analysis. A data matrix from the principal components of the essential oils detected in six samples was elaborated. Prior to principal component analysis (PCA) and cluster analysis, the variables were standardized for a normalized PCA. The set of data was processed through the commercial package Statistica. Euclidean distance was used to measure the similarity between samples, and the weighted pair-group average linkage method was used as an agglomerative

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algorithm. The PCA process established the combination of essential oil constituents or samples that account for the largest amount of variance to form the first principal component.

Plant Material. Single plant cuttings of *S. fruticosa* from a wild population in western Crete (Greece) were collected in the spring and put in a 25% perlite and 75% peat-composed rooting medium under mist propagation conditions for rooting. The wild plant material, used for hydroponical cultivation, was identified by Dr. D. Perdetzoglou (University of Athens), and a voucher specimen has been deposited in the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens (Greece).

Experimental Conditions. Three months after collection of the wild plant material, rooted cuttings were transferred to an NFT installation, and their apical shoots were pruned to accelerate the development of lateral ones. The NFT installation consisted of six independent NFT units, each one with header and catchment tanks and a bench-supported PVC gully (channel).

Three complete nutrient solutions were used, each one with a specific nitrogen, in the form of NO_3-N concentration (N1 = 100 mg/L, N2 = 150 mg/L, and N3 = 200 mg/L). The nutrient solution was recirculated in each channel at a flow rate of 3 L/min.The target pH value of the nutrient solutions N1, N2, and N3 was 6.0, whereas the target electrical conductivities were 1.2, 1.3, and 1.4 mS/cm, respectively. Both pH and electrical conductivity (EC) were monitored twice daily by means of portable pH and electrical conductivity meters and adjusted for each channel by adding a 5% nitric acid solution and the corresponding nutrient solution, respectively.

Chemical analyses of the nutrient solution were performed twice a week to keep constant nitrogen concentrations. The nutrient solutions were monthly renewed to avoid any possible nutrient deficiency or excess.

Two samplings were performed, at full bloom (flowering stage) and at the end of the seed formation stage. In each sampling, five randomly sampled plants were harvested from each channel. The freshly harvested shoots were then naturally dried under shade conditions so as to avoid essential oil losses.

Isolation of the Essential Oils. From the dried shoots, leaves were separated and a 10 g sample of them was distilled by using a Clevenger type apparatus. The essential oil obtained after 3.5 h of distillation from each sample was measured (milliliters) and collected in a small beaker to which a small amount of anhydrous sodium sulfate was added to absorb water; it was then kept in a stoppered vial at −15 °C for further analysis.

Gas Chromatography—Mass Spectrometry. The essential oils were analyzed using a capillary GC-MS system operating in EI mode. The use of a chiral column allowed the determination of enantiomers in several main compounds. In most cases, only one isomer was present in the essential oil, the other being absent or present in trace amounts.

GC-MS analyses were performed on a Hewlett-Packard 5973-6890 system operating in EI mode (70 eV) equipped with a split/splitless injector (220 °C), a split ratio of 1/10, using three different columns: a fused silica HP-5 MS capillary column [30 m × 0.25 mm (i.d.), film thickness = 0.25 μm]; an HP-Innowax capillary column [30 m × 0.25 mm (i.d.), film thickness = 0.50 μm]; and a chiral Cydex B (SGE Scientific Ltd.) capillary column [50 m × 0.22 mm (i.d.), film thickness = 0.25 μm]. The temperature program for the HP-5 MS column was from 60 °C (5 min) to 280 °C at a rate of 4 °C/min; that for the HP-Innowax column was from 60 to 260 °C at a rate of 3 °C/min; and that for the Cydex B column was from 50 to 130 °C (2 min) at a rate of 2 °C/min and from 130 to 250 °C at a rate of 4 °C/min. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The injection volume of each sample was 2 μL.

Identification of constituents was based on comparison of retention indices with those of authentic samples (in the case of compound 77 in **Table 2**) (*10*) and on the basis of their mass spectral fragmentation (*11*) and by using the Wiley 138 I/NBS, GC-MS spectrometry library.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils. The yields (v/w) of the essential oils from the air-dried leaves of the hydroponically cultivated *S. fruticosa* are shown in **Table 1**. In this table are presented different letters (a−d) following mean numbers, indicating statistically significant difference at the 0.05 level.

Table 1. Mean Leaf Essential Oil Content (Milliliters per 100 g) at Two Developmental Stages[a]

treatment	flowering stage	seed formation stage
N1	1.575d	2.375a
N2	1.775c	2.450a
N3	1.550cd	2.000b

[a] N1, N2, and N3 are the three different nitrogen concentrations (i.e., 100, 150, and 200 mg/L, respectively) used in the NFT experiments. Means followed by different letters are significantly different at the 0.05 level.

The chemical composition of the essential oils was analyzed by GC-MS [electron impact (EI)]. Qualitative and quantitative analytical results are shown in **Table 2**. Analysis of the constituents of the essential oils showed qualitative and quantitative differences due to the sampling in different periods as well as different concentrations of nitrogen in the nutrient solutions. 1,8-Cineole, β-caryophyllene, and viridiflorol were present in sufficient percentages in all cases with the exception of 1,8-cineole, which was totally absent in the case of full bloom and seed formation periods when 200 mg of N/L was used (**Table 2**). Tricyclene, α-fanchene, δ-2-carene, *p*-cymene, *cis*-linalool oxide, *cis*-piperitol, *trans*-carveol, thymol, δ-elemene, α-terpinyl acetate, epizonarene, α-muurolene, cadina-1,4-diene, β-calacorene, benzyl benzoate, farnesyl acetone, and a clerodane type diterpene were in most cases almost absent. 13-*epi*-Manool, a labdane type diterpene, has been identified in high percentages being predominant at the seed formation period when 200 mg of N/L was used. Its identification concerning the stereochemistry at C-13 has been achieved by using recently published results from our laboratory on the identification of the epimers of manoyl oxide using its mass spectrum fragmentation pattern (*11*).

It is noteworthy that compound 77 (**Table 2**) has never been reported in the genus *Salvia* and has been characterized as labd-7,13-dien-15-ol. Its identification has been made by using an authentic sample recently isolated in our laboratory (*10*). It is important to identify such rare constituents in essential oils or in extracts and use them as chemotaxonomic markers to distinguish species (*10*).

Leaf essential oil content increased with time for all of the treatments with nitrogen, the highest values being recorded at the end of the seed formation stage (**Table 1**).

The effect of nitrogen concentration was minor with a remarkable decrease for 200 mg of N/L (N3) (**Table 1**) at the end of the seed formation stage.

The decrease in essential oil content with the high nitrogen level N3 (200 mg/L) (**Table 1**) agrees with the results reported by Singh working with *Mentha arvensis* L. in sand culture (*12*). He found that the essential oil content increased with an increase in nitrate-N (NO_3-N) up to 16 mequiv/L, beyond which it decreased. Bhardwaj and Kaushal working with peppermint (*Mentha piperita* L.) in soil culture reported an increase in essential oil content with increased nitrogen levels up to 150 kg/ha (*13*), whereas they reported no effect with higher nitrogen doses. In another experiment, Singh (*12*) reported that for mint species, grown in soil, the essential oil content decreased with an increased nitrogen concentration from 0 to 50 to 100 kg/ha, the essential oil content being higher with 0 nitrogen applied under their experimental conditions. The gradual increase in

Table 2. Chemical and Percentage Composition of the Essential Oils of Leaves of Cultivated *S. fruticosa*

	compound[c]	RI[a]	**1**[b]	**2**	**3**	**4**	**5**	**6**
1	tricyclene	926	0.13					
2	α-thujene	931	0.12	0.15	0.11	0.06		
3	α-pinene	939	5.08	3.74	2.63	4.07		
4	α-fenchene	951				0.01		
5	camphene	953	0.56	0.62	0.42	0.75		
6	β-pinene	980	4.63	4.54	3.74	3.99		
7	myrcene	990	6.99	3.73	2.56	3.73		
8	δ-2-carene	1001				0.12		
9	α-phellandrene	1002	0.17					
10	α-terpinene	1014	0.33	0.27	0.25			
11	*p*-cymene	1026	0.11					
12	1,8-cineole	1028	26.76	37.47	28.58	22.51		
13	β-ocimene[c]	1044	0.03					
14	γ-terpinene	1062	0.67	0.35	0.42	0.47		
15	*cis*-sabinene hydrate	1068	0.10	0.22	0.19	0.19		
16	*cis*-linalool oxide (furanoid)	1074				0.01		
17	terpinolene	1088	0.22	0.18	0.16	0.21		
18	*trans*-sabinene hydrate	1097	0.06	0.10	0.08	0.08		
19	linalool	1098	0.11	0.09	0.11	0.0		
20	α-thujone	1102	0.65	0.97	1.06	0.86		
21	β-thujone	1114	0.25	0.43	0.44	0.42		
22	terpinen-1-ol	1130	0.04			0.05		
23	camphor	1142	0.42	1.51	1.15	1.60		
24	δ-terpineol	1148	0.87	1.32	1.01	1.32	1.42	1.62
25	terpinen-4-ol	1165	0.34	0.37	0.29	0.36		
26	α-terpineol	1177	2.54	3.61	3.03	3.76	6.58	6.23
27	α-phellandrene epoxide	1187	0.1					0.15
28	*cis*-piperitol	1193				0.01		
29	*trans*-carveol	1217				0.02		
30	(–)-bornyl acetate	1284	0.07	0.07	0.08	0.07		
31	thymol	1290				0.03		
32	carvacrol	1298	0.09			0.1	0.66	
33	δ-elemene	1134						0.1
34	*cis*-limonene oxide	1138	0.04					
35	α-terpinyl acetate	1350					0.1	
36	α-cubebene	1351	0.06	0.12	0.14	0.09		
37	eugenol	1356			0.10	0.10		
38	β-cubebene	1370		0.44	0.41			
39	(–)-isoledene	1373	0.31	0.23	0.21	0.25		
40	a-copaene	1376	0.08	0.10	0.10			
41	β-bourbonene	1380		0.08		0.03		
42	α-gurjunene	1409	0.27	0.35	0.31	0.12	1.18	
43	β-caryophyllene	1418	0.25	13.03	12.18	12.24	9.89	11.47
44	calarene	1432	0.29	0.25	0.44	0.27		
45	γ-elemene	1433				0.08		
46	(+)-aromadendrene	1439	4.53	3.15	3.02	3.57	2.83	2.84
47	α-guaiene	1439			0.38			
48	α–humulene	1454	6.02	4.76	4.75	4.94	10.19	8.59
49	*allo*-aromadendrene	1461	0.45	0.32	0.34	0.30		
50	γ-gurjunene	1473	0.12	0.08	0.08	0.09		
51	γ-muurolene	1478	0.17			0.2		
52	α-amorphene	1485	0.13	0.27	0.33	0.02	1.02	0.61
53	*cis*-β-guaiene	1490	0.1					
54	δ-selinene	1491			0.09	0.06		
55	ledene	1493	2.36	0.95	1.12	1.17	2.51	1.54
56	epizonarene	1497				0.45		
57	α-muurolene	1499				0.02		
58	γ-cadinene	1513		0.15	0.18	0.14		0.45
59	δ-cadinene	1524	0.33	0.4	0.46	0.37		1.06
60	α-cadinene	1534	0.15				0.1	
61	cadina-1,4-diene	1535				0.02		
62	β-calacorene	1563				0.03		
63	*epi*-globulol	1532	0.23	0.11	0.14	0.08	0.49	
64	(*Z*)-3-hexenylbenzoate	1570	0.18		0.17	0.07		
65	spathulenol	1576	0.12	0.18			1.13	0.87
66	(–)-caryophyllene oxide	1580	1.00	0.62	1.11	0.94	3.93	3.29
67	viridiflorol	1590	15.71	7.16	10.66	10.95	37.93	25.35
68	β-oplopenone	1606				0.03		
69	T-muurool	1641	0.09		0.07	0.07		
70	caryophylla-4(14),8(15) dien-5-β-ol	1642	0.11	0.10		0.32	0.65	
71	β-eudesmol	1649	0.1	0.1				
72	benzyl benzoate	1765				0.03		
73	2-pentadecanone, 6,10,14-trimethyl-	1845				0.05		
74	farnesyl acetone	1918				0.02		
75	biformene		0.18	0.08	0.15	0.07		
76	13-*epi*-manool	2056	11.39	4.61	13.15	12.87	14.35	25.74
77	labd-7,13-dien-15-ol	2280	1.09	0.36	1.06	1.02	1.18	0.61
78	clerodane type diterpene					0.1		
79	*trans*-ferruginol	2330	0.21		0.26	0.43		0.88
	total identified (%)		97.51	97.74	97.72	96.5	96.14	91.40

[a] RI (retention indices) on DB-5 column were calculated according to Van den Dool and Kratz (*15*). [b] **1**,**2** and **3**,**4** and **5**,**6**: essential oils of leaves of cultivated *S. fruticosa*, at full bloom (**1**, **3**, **5**) and at the end of seed formation (**2**, **4**, **6**) stages, using 100, 150, and 200 mg/L nitrogen, respectively. [c] Correct isomer not identified. [d] Compounds are listed according to their t_R on the DB-5 column and have been identified by using the respective standard compounds (no. **30**, **39**, **46**, **66**, and **77**). Retention indices and MS database have been used to tentatively identify the rest of the compounds in **Table 2**.

essential oil content from the full bloom (flowering stage) to the seed formation stage (**Table 1**) is in agreement with the results reported by Putievsky (*2*, *14*) for sage (*Salvia officinalis* L. and *Salvia fruticosa*), respectively, stating that there was an increase in essential oil content from March to August.

In a previous experiment we found (*6*) no effect of NO_3-N level at similar concentrations (100, 150, and 200 mg/L) on the essential oil content (percent) of leaves and bracts of *Origanum dictamnus* L. grown with NFT.

Results obtained from the PCA (**Figure 1**) and cluster analysis (**Figure 2**) showed the existence of two well-defined groups. The first one was defined by the samples **5**,**6** (concentration at 200 ppm) and the second one by two subclusters: the first with the samples **1**,**2** (concentration at 100 ppm) and the second with the samples **3**,**4** (concentration at 150 ppm). We applied the test of correlation coefficient (**Table 3**) to the data of the **Table 2** to clarify the possible linear correlations between the constituents of the studied samples. As can be seen, there is a strong significant positive correlation ($r = 0.99$) between the constituents of the samples **3**,**4** (concentration at 150 ppm),

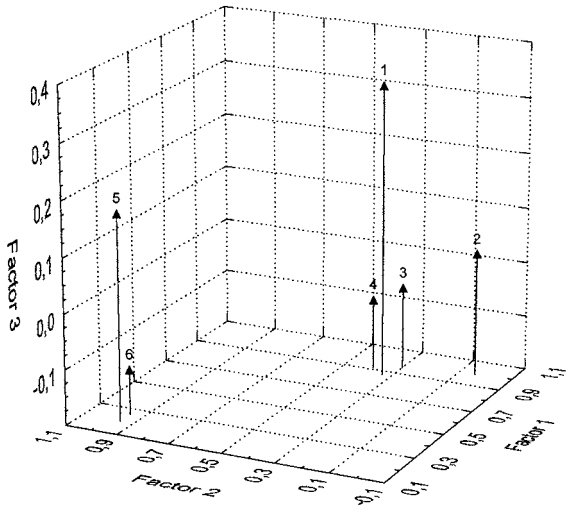

Figure 1. Relative position of samples **1**–**6** in the space defined by the three principal components.

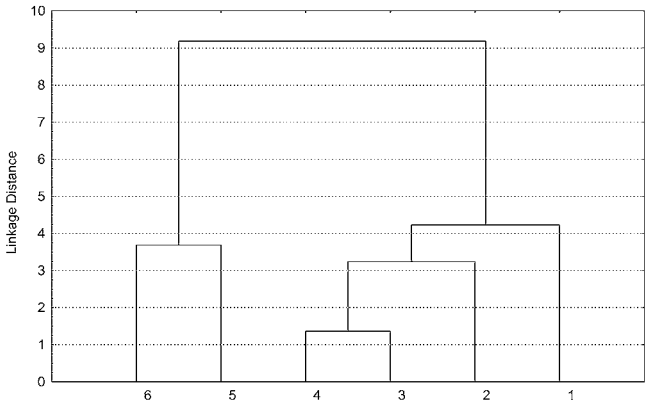

Figure 2. Two-dimensional dendrogram obtained in the cluster analysis of the essential oils of six samples of *S. fruticosa* based on the data (**Table 2**): horizontal, samples analyzed; vertical, differentiation level between samples.

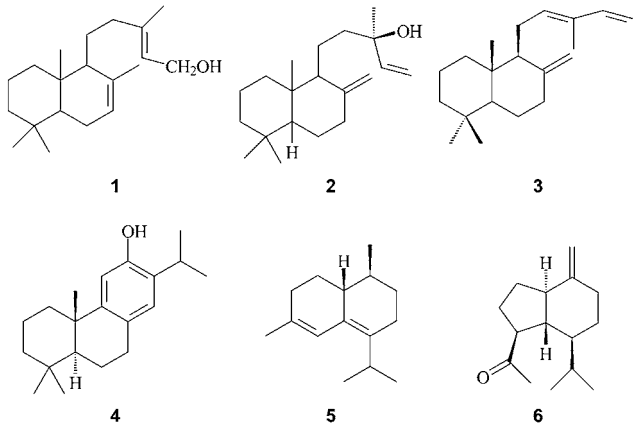

Figure 3. Structures of some uncommon terpenes identified in the essential oils of *S. fruticosa*: **1**, labd-7,13-dien-15-ol (no. 77); **2**, 13-*epi*-manool (no. 76); **3**, biformene (no. 75); **4**, *trans*-feruginol (no. 79); **5**, epizoranene (no. 56); **6**, β-oplopenone (no. 68).

Table 3. Pearson's Correlation Coefficient (Significant Level: $p < 0.05$)

variable	1[a]	2	3	4	5	6
1	1.00	0.86	0.91	0.91	0.52	0.52
2	0.86	1.00	0.95	0.91	0.26	0.27
3	0.91	0.95	1.00	0.99	0.46	0.53
4	0.91	0.91	0.99	1.00	0.54	0.62
5	0.52	0.26	0.46	0.54	1.00	0.91
6	0.52	0.27	0.53	0.62	0.91	1.00

[a] **1**,**2** and **3**,**4** and **5**,**6**: essential oils of leaves of cultivated *S. fruticosa*, at full bloom (**1**, **3**, **5**) and at the end of seed formation (**2**, **4**, **6**) stages, using 100, 150, and 200 mg/L nitrogen, respectively.

which is the highest degree of correlation, both with concentration at 150 ppm, as well as between the constituents of the samples **2**,**3** ($r = 0.95$), the first belonging to the concentration at 100 ppm and the second to the concentration at 150 ppm. It is important that the correlations between each of the samples **5**,**6** (concentration at 200 ppm) and all other samples represent the smaller degree of correlations. Moreover, we note the high degree of correlation between the following samples: **3**,**4** ($r = 0.99$), **5**,**6** ($r = 0.91$), **1**,**2** ($r = 0.86$)—all of the samples of the pairs belong to the same group of concentration and the smallest one is between samples **2** and **5** ($r = 0.26$). From the above results we can conclude that the NFT which was applied for *S. fruticosa* is a potent methodology for growing *Salvia* species. However, this approach opens an avenue for the development of NFT studies and for the production of aromatic and medicinal plants with better biological profiles.

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