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Effect of Phosphorus Concentration of the Nutrient Solution on the Volatile Constituents of Leaves and Bracts of *Origanum dictamnus*

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The chemical composition of the essential oils obtained from the leaves and bracts of hydroponically cultivated *Origanum dictamnus* were analyzed by GC−MS techniques. Three different concentrations of phosphorus (5, 30, and 60 mg/L) in the nutrient solution were used for the cultivation, using the nutrient film technique (NFT). A total of 46 different compounds were identified and significant differences (qualitative and quantitative) were observed between the samples. Carvacrol and *p*-cymene were identified as the main compounds in all samples analyzed, whereas thymoquinone was found in higher percentage in the leaves than in bracts. The essential oils were tested for their antibacterial activity against Gram-positive and Gram-negative bacteria. The oils obtained from the bracts were found to be more active. The results obtained from GC−MS analyses were submitted to chemometric analysis.

KEYWORDS: ***Origanum dictamnus*** **essential oils; hydroponic cultivation; antibacterial activity; GC−MS; chemometrics**

INTRODUCTION

Origanum dictamnus L. (Lamiaceae) or Cretan dittany is a white lanate subshrub, endemic to Crete (Greece), with egg-shaped or rounded leaves, loose terminals of pink flowers, and large purple bracts unique in the genus for having branched hairs. The plant grows wild on the rocky slopes of mountainous Crete (*1*, *2*). However, it is also cultivated for its therapeutic properties (*3*), which have been known since antiquity. It is a well-known plant in Crete under the names dictamos and erontas. It is mainly consumed as a herbal tea, because of its remedial properties, such as anti-hemorrhage, healing wounds and gastric ulcers, facilitating childbirth and abortion, and stimulating the nervous system (*3*, *4*). The essential oil obtained from a mixture of leaves and bracts of the wild plant has been studied (*5*−*9*). Recently, we have published the chemical composition of the volatile constituents of bracts and leaves of wild and hydroponically cultivated plant using three different concentrations of nitrogen in the nutrient solution (*10*). The information available concerning the hydroponic cultivation of aromatic and medicinal plants is generally limited. However, Davtyan (*11*) reported substrate cultivation of aromatic and medicinal plants out-yielded soil grown plants. The same author reported that, in the case of *Pelargonium roseum* Willd. and *Ocimum basilicum* L., the essential oil content in hydroponically grown plants was higher than that of soil grown plants. The nutrient film technique (NFT) is widely used for commercial vegetable production (*12*), but also has an excellent application

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Table 1. Yield % (v/w) of the Essential Oils of Leaves and Bracts of Cultivated *Origanum dictamnus*

phosphorus concentration mg/L	leaves	bracts
5	3.1	3.8
30	2.7	4.0
60	2.8	4.3

in plant nutrition studies (*13*). To evaluate the performance of selected common herbs grown with the NFT, a series of experiments was carried out, and it was established that dittany plants could be successfully grown, and furthermore, that the yield was remarkable (*14*, *15*). Continuing our research on the hydroponically cultivated *O. dictamnus*, we now report the results obtained in an investigation on the qualitative and quantitative analysis of the essential oils of *O. dictamnus* using three different concentrations of phosphorus in the nutrient solutions in order to test whether the oil yield and chemical composition were affected. The knowledge of the composition of the essential oil of *O. dictamnus* is of great importance for understanding the conditions under which the hydroponically cultivated plants could generate qualitatively better high yielding oils. The results were compared with those recently published by us (*10*) when three different concentrations of nitrogen were used in the nutrient solutions for cultivation of *O. dictamnus*.

MATERIALS AND METHODS

Chemometric Statistical Analysis. Data of **Table 2** were submitted to cluster analysis in order to clarify the phenotypic relationships of the samples. Prior to cluster analysis, the variables were standardized for a normalized procedure. The set of data was processed through STATISTICA commercial statistical package. City-block (Manhattan, method to measure the distance among the individuals, i.e., samples of plants) was used to measure the similarity between samples of bracts and leaves of cultivated *Origanum dictamnus*, and Ward's linkage method was used as an agglomerative algorithm (amalgamation joining rule). Moreover, we applied the test of correlation coefficient to the **Table 2** data to clarify the possible linear correlation between the studied samples of bracts and leaves.

Plant Material. The leaves and bracts from the cultivated *O. dictamnus* were collected at the Institute of Subtropical Plants and Olive Trees in Chania (Crete). The wild *O. dictamnus*, used for the hydroponical cultivations, was identified by Dr. D. Perdetzoglou (University of Athens) and a voucher specimen (Perdetzoglou and Demetzos, no 1643A, ATHU) has been deposited in the Laboratory of Pharmacognosy, University of Athens (Greece).

Experimental Conditions. The cultivation was carried out in an unheated glasshouse, using shoot cuttings, which were obtained from a single dittany plant of the same wild population which was recently used (*10*). The NFT system, originally designed for lettuce cultivation, has been previously described (*16*). Each treatment occupied two NFT units (channels), the second being a replicate. In each unit the nutrient solution, with a constant volume of 90 L, was recirculated by a submersible pump at a rate of 1800 mL/min.

Three levels of phosphorus i.e 5, 30, and 60 mg/L were applied, and the nutrient solutions used were complete with nitrogen (150 mg/L) and potassium (150 mg/L). The solutions were analyzed weekly for phosphorus, nitrogen, and potassium, with the appropriate adjustments made when necessary. To maintain a good equilibrium of nutrients the solutions were renewed every two weeks. A pH of 6 was maintained by addition of nitric acid. Likewise, the electrical conductivity target value was 2.0 mS/cm, which was adjusted by the daily addition of the complete stock nutrient solution.

Shoot cuttings (7−10 cm long) from dittany plants were placed under mist propagation conditions. After a month, 32 rooted cuttings, selected for their uniformity, were placed 25 cm apart in each of the NFT channels. Two channels were used, as replicates, for each of the three examined phosphorus levels (i.e. 5, 30, and 60 mg/L). In July, when all the plants were at the flowering stage, 10 plants from each treatment were sampled. Their shoots were weighed and placed in an air oven at 40 °C until they had reached a constant weight. The dried shoots of the plants belonging to the same treatment were mixed, and the bracts were separated from the leaves.

Isolation of the Essential Oils. The leaves and bracts were subjected to separate hydrodistillation for 3 h. All the oils obtained were dried over anhydrous Na_2SO_4 and stored under refrigeration (4 °C).

Gas Chromatography−Mass Spectrometry. The essential oils were analyzed using a capillary GC−MS system operating in the EI mode. The GC−MS analyses were carried out using a Hewlett-Packard (HP) 5973 mass selective detector, using two different columns: a fused silica HP-5 MS capillary column (30 m × 0.25 mm (i.d.), film thickness 0.25 μm) and a HP-Innowax capillary column (30 m × 0.25 mm (i.d.), film thickness 0.50 μm). The temperature program for the HP-5 MS column was from 50 °C (5 min) to 280 °C at a rate of 3 °C/min; for the HP-Innowax column it was from 60 °C to 260 °C at a rate of 3 °C/min. Mass unit conditions were the following: ion source 230 °C, ionization energy 70 eV, electron current 1435 μA. Identification of the components was based on comparison of their mass spectra with those of Wiley 275 and NBS Libraries (*17*) and those described by Adams (*18*), as well as by comparison of their retention indices with literature data (*18*, *19*). In many cases, the essential oils were subjected to co-chromatography with authentic compounds (Fluka, Sigma).

Antimicrobial Activity. Each essental oil was dissolved at 10 mg/mL with dimethyl sulfoxide (DMSO) and diluted with tryptone soya broth (TSB, Oxoid CM 129). Final concentrations were 1000, 500, 250, and 125 μg/mL in the medium, and final concentration of DMSO was 1% in the medium (*20*).

Three Gram-positive bacteria (*Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 10907), and *Micrococcus flavus* (ATCC 10240)) and three Gram-negative bacteria (*Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (clinical isolates), and *Escherichia coli* (ATCC 35218)) were used in the present study.

Bacterial species were cultured overnight at 37 °C in TSB, and suspensions containing ~10^9 cells/mL were obtained.

To obtain quantitative data, the modified micro-dilution technique was used (*21*, *22*). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 × 10^5 in a final volume of 100 μL per well. The inocula were stored at +4 °C for further use. Dilutions of the inocula were cultured on tryptone soya agar (TSA, Oxoid CM 131) to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtiter plates. The micro plates were incubated for 48 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations which completely inhibited bacterial growth (MICs).

DMSO was used as a control and streptomycin was used as a positive control.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils. The yields (v/w) of the essential oils from the air-dried leaves and bracts of the cultivated *O. dictamnus* are shown in **Table 1**.

The chemical composition of the essential oils was analyzed by GC−electron impact (EI) MS. Qualitative and quantitative analytical results are shown in **Table 2**. **Figure 1** summarizes the data in **Table 2**.

The analysis of the constituents of the oils of the leaves and bracts showed qualitative and quantitative differences due to the different concentrations of phosphorus in the nutrient solutions. We have to notice that some compounds identified in leaves (i.e., *p*-mentha-1,3,8-triene, *p*-cymen-8-ol, 6-methyl-3,5-heptadien-2-one, α-cubebene, α-humulene, γ-muurolene,

Table 2. Chemical and Percentage Composition of the Essential Oils of Leaves and Bracts of Cultivated *Origanum dictamnus*

			leaves			bracts		
compound[c]		RI[a]	**1**	**2**	**3**	**4**	**5**	**6**
1	α-thujene	931	1.41	1.70	1.46	0.91	0.59	0.61
2	α-pinene	939	1.00	1.27	1.09	1.09	0.73	0.43
3	camphene	953	0.53	0.72	0.56	0.18	0.13	0.08
4	β-pinene	980	0.24	0.27	0.23	0.23	0.16	0.12
5	1-octen-3-ol	978	0.32	0.29	0.30	0.27	0.22	0.22
6	myrcene	991					0.23	1.03
7	3-octanol	993	0.19	0.17	0.16	0.10	0.07	0.08
8	α-phellandrene	1005				0.01		0.10
9	δ-3-carene	1011	0.10	0.10	0.10	0.12	0.09	0.08
10	α-terpinene	1018	0.24	0.41	0.19			0.22
11	*p*-cymene	1026	41.46	42.41	42.18	26.44	18.68	11.13
12	β-phellandrene	1031	0.57		0.58	0.50		
13	γ-terpinene	1062				0.04	0.07	4.62
14	*cis*-sabinene hydrate	1068	1.26	0.05	1.23	1.04	0.98	0.66
15	terpinolene	1088						0.07
16	*trans* -sabinene hydrate	1097	0.34	0.09	0.31	0.35	0.05	
17	*p*-mentha-1,3,8-triene	1111	0.06	0.08				
18	*trans*-sabinol	1140			0.03			
19	*trans* -verbenol	1144			0.04			
20	borneol	1165	1.36	1.11	1.09	0.25	0.11	
21	terpinen-4-ol	1177	0.59	1.34	0.51	0.23	0.33	0.08
22	p-cymen-8-ol	1183	0.09	0.17	0.14			
23	6-methyl-3,5-heptadien-2-one		0.13		0.19			
24	α-terpineol	1174	0.12	0.12			0.05	
25	cis-dihydrocarvone	1193		0.04				
26	cis-carveol	1229					0.20	
27	carvone	1242		0.09	0.09		0.23	
28	thymoquinone	1249	10.80	13.59	12.59		0.28	
29	thymol	1290	0.28	0.24	0.36	0.32	0.29	10.83
30	carvacrol	1298	34.83	29.11	31.73	63.87	73.41	60.04
31	α-cubebene	1351	0.19	0.12	0.11			
32	cis-carvyl acetate	1362						0.14
33	α-copaene	1376	1.58	1.01	0.97	0.20		0.16
34	β-caryophyllene	1418	0.64	1.27	0.55			0.07
35	α-humulene	1454		0.09	0.04			
36	β-cubebene	1390		0.04				
37	γ-muurolene	1477	0.10	0.16				
38	germacrene D	1480		0.04				
39	β-bisabolene	1509		0.04				
40	γ-cadinene	1513		0.14	0.11			
41	*cis*-β-guaiene	1500					0.04	
42	δ-cadinene	1524	0.22	0.19	0.15		0.05	0.07
43	β-sesquiphellandrene	1524	0.19	0.58				
44	caryophyllene oxide	1580	0.93	0.93	0.87	0.78	1.31	1.50
45	6,10,14-trimethyl-2-pentadecanone						0.03	
46	abietatriene	2054					0.06	0.13
	total identified (%)		99.77	97.98	97.96	96.93	98.39	92.47
	$[\alpha]_D^{20}$		–9.67 (pentane, *c* 0.1)	–0.37 (pentane, *c* 3.3)	–1.1 (pentane, *c* 0.4)	–0.6 (pentane, *c* 0.5)	–0.53 (pentane, *c* 0.6)	–17.69 (pentane, *c* 0.2)

[a] RI (Retention indices) on DB-5 column were calculated according to Van den Dool and Kratz (*24*). [b] **1**, **2**, **3** and **4**, **5**, **6**: Essential oils of leaves and bracts of cultivated *O. dictamnus*, using 5, 30, and 60 mg/L phosphorus, respectively. [c] Compounds are listed according to their R_t to the DB-5 column.

germacrene D, γ-cadinene, and β-sesquiphellandrene) were not present at all in bracts. Thymoquinone was present in sufficient and almost equal percentages in leaves of plants grown in the three phosphorus concentrations used, contrary to that of bracts (**Table 2**). The essential oils were characterized by the presence of carvacrol and *p*-cymene, which were the predominant compounds in all cases, were present in higher percentages in the bracts than in the leaves, and in the leaves than in the bracts, respectively. Myrcene, α-phellandrene, γ-terpinene, terpinolene, *trans*-sabinol, *trans*-verbenol, *cis*-dihydrocarvone, *cis*-carveol, carvone, *cis*-carvyl acetate, γ-muurolene, *cis*-β-guaiene, and 6,-10,14-trimethyl-2-pentadecanone were in most cases almost absent from the leaves and bracts.

In all cases, the yield of the essential oils in the present study seems to be equal to that obtained from cultivated *O. dictamnus* using nitrogen in nutrient solutions, but greater than that obtained from leaves and bracts of wild-growing *O. dictamnus* (*10*).

Analysis of the essential oils of *O. dictamnus* cultivated using different concentrations of phosphorus showed 46 identified compounds, in contrast to the 25 compounds found in the cultivated and wild *O. dictamnus* (*10*). The percentage content of carvacrol in the bracts remained higher than that in the leaves and was comparable to that determined in the wild plant as well as in cultivated plants using nitrogen instead of phosphorus nutrient solutions.

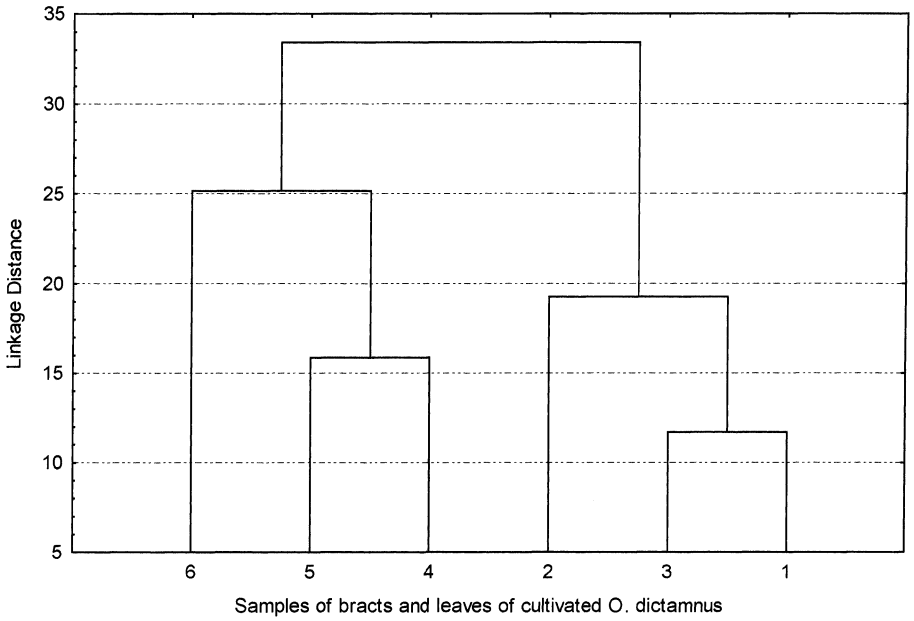

Figure 1. Two-dimensional dendrogram obtained in the cluster analysis of the essential oils of bracts and leaves of cultivated *Origanum dictamnus* based on the data (**Table 2**): horizontal, samples of bracts and leaves analyzed; vertical, differentiation level between samples.

Table 3. Antimicrobial Activity of the Essential Oils of Cultivated *Origanum dictamnus*

	leaves			bracts			
bacteria	**1**[a]	**2**	**3**	**4**	**5**	**6**	**S**
Staphylococcus aureus (ATCC 6538)	500		500	250	500	600	100
Bacillus subtilis (ATCC 10907)							100
Micrococcus flavus (ATCC 10240)				500			100
Pseudomonas aeruginosa (ATCC 27853)				500	500	500	500
Proteus mirabilis (clinical isolates)		500	500	125	500	500	400
Escherichia coli (ATCC 35218)		500		125	250	500	200

[a] **1**, **2**, **3** and **4**, **5**, **6**: Essential oils of leaves and bracts of cultivated *O. dictamnus*, using 5, 30, and 60 mg/L phosphorus, respectively. [b] S, Streptomycin.

In the present study, the compounds tricyclene, sabinene, limonene, linalool, and camphor were not identified, contrary to the results published from the analysis of the wild *O. dictamnus* and of cultivated *O. dictamnus* using nitrogen in nutrient solution (*10*). From the above results, we can conclude that the NFT technique is a potent methodology for growing *O. dictamnus* successfully from which a remarkable oil yield was obtained. Concerning the main identified constituents, carvacrol remains in high percentage (29.11−73.41%) in all samples, and the percentage of *p*-cymene was higher compared to that of the published data (*10*). An increase in the percentage of *p*-cymene accompanied by a decrease in the percentage of carvacrol was observed when phosphorus was used in nutrient solution, especially in the case of leaves (**Table 2**), compared to that when nitrogen was used (*10*). It is noteworthy that new constituents were identified (**Table 2**) which have not been reported in previous studies (*5*, *10*), and it may be possible that the phosphorus present activated enzymes which were less active in the case when nitrogen was used in the nutrient solution or in the case of wild plants.

The results of the antibacterial activity (**Table 3**) showed that the oils of the bracts proved to be more active than the oils of the leaves. It is noteworthy that the oils of *O. dictamnus* cultivated using phosphorus in the nutrient solution were characterized by the presence of a high percentage of carvacrol, and therefore it seems possible that the significant antibacterial activity could to a considerable degree be attributed to its known biological properties (*23*).

Table 4. Pearson's Correlation Coefficient

variable	**1**[a]	**2**	**3**	**4**	**5**	**6**
1	1.00	1.00*[b]	1.00*	0.89*	0.99*	0.88*
2	1.00*	1.00	1.00*	0.99*	0.99*	0.88*
3	1.00*	1.00*	1.00	0.88*	0.99*	0.88*
4	0.89*	0.89*	0.89*	1.00	0.90*	0.95*
5	0.99*	0.99*	0.99*	0.90*	1.00	0.86*
6	0.88*	0.88*	0.88*	0.95*	0.86*	1.00

[a] **1**, **2**, **3** and **4**, **5**, **6**: Essential oils of leaves and bracts of cultivated *O. dictamnus*, using 5, 30, and 60 mg/L phosphorus, respectively. [b]Correlations marked * are significant at $p < 0.500$.

Results obtained from correlation analysis (**Table 4**) showed that each one of the bract samples had the same degree of correlation with the whole set of the data of the leaves, respectively. Moreover, there is a strong positive linear correlation between the constituents of the samples of the leaves ($R = +1$; significant at $p < 0.05$). In conclusion, the samples obtained from the leaves seem to be more constant than those from the bracts.

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