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ESSENTIAL OILS AND HYDROCARBONS FROM LEAVES AND CALLI OF *ORIGANUM VULGARE* SSP. *VIRENS*

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Key Word Index—*Origanum vulgare* ssp. *virens*; Lamiaceae calli; essential oils; hydrocarbons; *n*-alkanes; monoterpenoids; sesquiterpenoids; naphthalene.

Abstract—Green friable calli (G-calli) and dark abnormal root primordia containing calli (R-calli) of *Origanum vulgare* ssp. *virens* were induced from leaves and established in the presence of 0.25 mg and 1 mg l^{-1} (2,4-D), respectively. Leaves, of the same type of those used in the calli induction, G-calli and R-calli were submitted to hydrodistillation and the respective hydrodistillates were analysed by GC and GC-mass spectrometry. The hydrodistillate from leaves consisted of *ca* 50% monoterpenoids (35.2% oxygenated monoterpenes and 14.3% monoterpene hydrocarbons) and *ca* 40% of sesquiterpenoids (2.5% oxygenated sesquiterpenes and 37.4% sesquiterpene hydrocarbons). Linalool (16.4%) and (*E*)-β-ocimene (6.6%) were the major oxygenated monoterpene and monoterpene hydrocarbon, respectively. Globulol (0.94%) and δ-elemene (12.85%) were the major oxygenated sesquiterpene and sesquiterpene hydrocarbon, respectively. *n*-Alkanes, namely pentacosane (0.97%), heptacosane (0.9%) and nonacosane (1.0%), were also present. Hydrodistillates from G- and R-calli did not contain either mono- or sesquiterpenoids; *n*-alkanes were the main compounds found. The alkane concentration in R-calli was more than twice that of G-calli. Naphthalene (0.5 μg g^{-1} dry wt) and eicosane, (0.9 μg g^{-1} dry wt) produced by R-calli were absent in G-calli. Squalene concentration in G-calli was 6.7 fold greater than that found in R-calli.

INTRODUCTION

The composition of the essential oils of *Origanum* species have been studied by several authors [1–6]. Some have reported variations in yield and composition according to genetic characteristics [7], phase of life-cycle [8, 9] and geographic origin [5, 6]. Studies on production of essential oils by *in vitro* cultures of *Origanum* species are scarce. Although *in vitro* cultures of *O. vulgare* shoots have been established [10, 11], reports on the production of essential oils by such systems are absent. However, according to Svoboda *et al.*[12], accumulation of small amounts of volatile oil, not associated to differentiation of compartments, occurs in *O. vulgare* calli.

Origanum vulgare L. grows wild in the National Park of Peneda Gerês in the north of Portugal and has been used for a long time by people of that region as an aromatic and medicinal plant. To our knowledge, determination of the composition of essential oils, as well as the establishment of *in vitro* cultures from *O. v.* ssp. *virens* plants of this ecotype has not yet been performed. In the present paper, we compare the composition of hydrodistillates from leaves of *in vivo O. v.* ssp. *virens* plants with those of calli induced from the same type of leaves.

RESULTS AND DISCUSSION

The essential oil isolated from leaves of *O. v.* ssp *virens* was a complex mixture with more than 70 components, 50 of which were identified, corresponding to 80% of the total oil. The identified components with their percentages and retention times on a DB-5 column are listed in Table 1. Most of the compounds were hydrocarbons (14.3% monoterpene hydrocarbons; 37.4% sesquiterpene hydrocarbons and 2.8% alkanes). The mass spectra of compounds 22 and 30 are both consistent with sesquiterpene hydrocarbons. (*E*)-β-Ocymene (6.57%) was the major monoterpene hydrocarbon and δ-elemene (12.85%), together with β-caryophyllene (11.07%), were the major sesquiterpene hydrocarbons. Similar total relative amounts of monoterpene hydrocarbons are reported for flowering parts of *O. majorana* [1] and *O. onites* [3]. Higher relative amounts of these type of compounds were reported, however, for *O. vulgare* ssp.

Table 1. Composition of essential oil from leaves of *Origanum vulgare* ssp. *virens* collected in Natural Park of Peneda Gerês

Compound no.	Name	Retention Time (min)	Leaves (%)
1	Nonane	4.05	t
2	α-Pinene	4.70	t
3	Camphene	5.05	0.20
4	Sabinene	5.57	t
5	Octen-3-ol	5.70	0.37
6	3-Octanone	5.85	0.27
7	Myrcene	5.95	3.37
8	3-Octanol	6.07	0.28
9	α-Terpinene	6.57	t
10	Limonene	6.88	0.48
11	(*Z*)-β-Ocimene	7.12	3.70
12	(*E*)-β-Ocimene	7.35	6.57
13	γ-Terpinene	7.63	t
14	Terpinolene	8.38	t
15	Linallool	8.37	16.36
16	Unknown	9.55	9.59
17	Borneol	10.63	t
18	4-Terpineol	10.92	t
19	α-Terpineol	11.30	9.20
20	Linalyl acetate	13.12	t
21	Isobornyl acetate	13.97	t
22	Unknown	15.12	1.30
23	δ-Elemene	15.37	12.85
24	Neryl acetate	16.10	t
25	α-Copaene	16.42	t
26	Geranyl acetate	16.63	t
27	β-Bourbonene	16.67	t
28	β-Elemene	16.85	0.35
29	β-Caryophyllene	17.58	11.07
30	Unknown	17.95	2.64
31	α-Humulene	18.47	1.32
32	Germacrene-D	19.20	0.98
33	Germacrene-B	19.67	6.58
34	α-Farnesene*	19.85	t
35	δ-Cadinene	20.28	0.34
36	(*E*)-γ-Bisabolene	20.57	t
37	Ledol	21.38	t
38	4-β-Hydroxygermacra-1(10),5-diene	21.58	0.70
39	Spathulenol	21.67	0.46
40	Caryophyllene oxide + Globulol	21.82	0.94
41	*tau*-Cadinol	23.15	t
42	*tau*-Muurolol	23.22	t
43	α-Cadinol	23.52	0.37
44	Tricosane	36.27	t
45	Tetracosane	37.97	t
46	Pentacosane	39.63	0.97
47	Hexacosane	41.25	t
48	Heptacosane	42.82	0.89
49	Squalene	44.90	0.52
50	Nonacosane	45.87	0.97

*Exact isomer not determined.

hirtum and *O. syriacum* [3]. Sesquiterpene compounds, occurred only in low amounts in flowering parts of *O. onites*, *O. syriacum* and of some sub-species of *O. vulgare*, namely *O. v.* ssp. *glandulosum* [6] and *O. v.* ssp. *hirtum* [3, 6]. In flowering parts of some biotypes of *O. v.* ssp. *vulgare* and *O. v.* ssp. *virens*, however, β-caryophyllene amounts to over 15% of the essential oil [6].

The total oxygenated components represented *ca* 38.5% of the total oil of leaves of *O. v.* ssp. *virens* (oxygenated monoterpenes, 35.2%; oxygenated sesquiterpenes, 2.5%). Compound 16 (Table 1) showed a mass spectrum characteristic of an oxygenated monoterpene being consistent with a terpineol isomer. Linalool (16.4%) was the major oxygenated monoterpene and globulol (0.9%) was the main oxygenated sesquiterpene (Table 1). The oxygenated monoterpene, carvacrol, which represents more than 50% of the total essential oil of flowering parts of *O. majorana* [1] and more than 60% of those of *O. onites*, *O. syriacum*, *O. v.* ssp. *hirtrum* [3] and *O. v.* ssp. *glandulosum* [6] was not detected in leaves of *O. v.* ssp. *virens*. The same holds true for the isomer, thymol, which accounts for more than 40% in essential oils of inflorescences of some biotypes of *O. v.* ssp. *hirtum* and *O. v.* ssp. *gracile* [6], and for *p*-cymene and γ-terpinene, the two precursors of the thymol and carvacrol, which are the main monoterpene hydrocarbons in essential oils of flowering parts of *O. majorana* [1]. *O. onites*, *O. syriacum* [3], *O. v.* ssp. *hirtum* [3, 6], *O. v.* ssp. *glandulosum* and *O. v.* ssp. *gracile* [6]. Amounts of thymol and carvacrol lower than 0.5% and amounts of *p*-cymene and γ-terpinene lower than 0.1% were reported for essential oils of inflorescences of some biotypes of *O. v.* ssp. *virens* [6]. In common with the essential oils of inflorescences of the same *O. v.* ssp. *virens* biotypes [6], the composition of the essential oil of leaves, reported, herein has high percentages of linalool and terpineol. Within the *O. vulgare* species, such high percentages of terpineol and linalool seems to be characteristic of the subsp. *virens*, since these compounds are essentially absent or present in trace amounts in the other subsp. of *O. vulgare* [6]. However, the composition of the essential oils may be greatly influenced by environmental factors. Linalool has been considered to play a role in the attraction of bees to flowers [13–15] and, as other essential oil compounds, linalool is induced systemically and released as a defence chemical in some plants after being attacked by herbivores [16, 17]. Similar results were reported for (*E*)-β-ocimene, one of the major monoterpene hydrocarbons found in the essential oil from leaves of *O. v.* ssp. *virens*, which is produced in significantly larger amounts in apple fruitlets after infestation by sawfly larvae [18]. In view of these reports, although some common essential oil characteristics can be envisaged for plants of *O. v.* ssp. *virens*, it is not surprising that the composition of the essential oil of different biotypes of this sub-species reveal great differences depending on the environmental factors.

Table 2. Hydrocarbons and fatty acids from calli of *Origanum vulgare* ssp. *virens*

Compound	Retention time (min)	G-Calli ($\mu g\,g^{-1}$ dry wt)	R-Calli ($\mu g\,g^{-1}$ dry wt)
Undecane	8.63	t	t
Naphthalene	11.00	—	0.5
Tetradecane	16.90	—	t
Hexadecane	21.94	t	0.2
Octadecane	26.40	t	0.2
Myristic acid	27.05	t	—
Nonadecane	28.53	t	0.7
Eicosane	30.66	—	0.9
Palmitic acid	31.12	0.6	—
Heneicosane	32.60	t	0.5
Docosane	34.48	0.5	0.7
Tricosane	36.27	0.6	0.7
Tetracosane	37.97	0.6	0.6
Pentacosane	39.63	t	0.5
Hexacosane	41.25	0.4	t
Squalene	44.90	8.7	1.3

Leaves of *O. v.* ssp. *virens* collected in May and cultured on MS medium supplemented with $1\,mg\,l^{-1}$ 2,4-D and $1\,mg\,l^{-1}$ benzyladenine (BA) showed the highest rate of calli induction (78% of total leaf segments used in the initiation of the cultures). Lower rates of calli induction were obtained using B5 medium supplemented with $1\,mg\,l^{-1}$ 2,4-D and 0.5 or $1.0\,mg\,l^{-1}$ BA (68% and 65%, respectively) and by using leaves collected in July (29%). Although, in many cases, the primary calli induced from leaves became dark, they were not necrotic. Transfer and the subculture of these calli to MS medium supplemented with $0.25\,mg\,l^{-1}$ 2,4-D and $0.5\,mg\,l^{-1}$ BA developed friable green calli (G-calli). The same type of primary calli when transferred and subcultured on MS medium supplemented with $1\,mg\,l^{-1}$ 2,4-D and $0.5\,mg\,l^{-1}$ BA developed dark calli with abnormal root primordia (R-calli).

With the exception of a few examples, the lack of success in *de novo* production of essential oil flavours, namely monoterpene and sesquiterpene compounds, by undifferentiated plant tissue cultures has been extensively reported [19–23]. This rule applies also to the calli of *O. v.* ssp. *virens* reported herein, whose hydrodistillates did not contain either monoterpene or sesquiterpene compounds. Squalene, a triterpene precursor of steroids, was the only terpene compound found (Table 2). According to some authors, the rapid degradation of monoterpene compounds and the lack of physical separation between their synthesis and storage may be responsible by their absence in undifferentiated tissues [23].

The majority of the compounds found in hydrodistillates of *O. v.* ssp. *virens* calli were alkanes whose composition and concentration differed markedly from those of leaves. Significant differences in alkane composition and concentration were also found between G-calli and R-calli (Table 2). *n*-eicosane, the major alkane present in R-calli was absent in G-calli. *n*-Nonadecane, *n*-heneicosane and *n*-pentacosane were present at concentrations of $0.7\,\mu g\,g^{-1}$ dry wt, $0.5\,\mu g\,g^{-1}$ dry wt and $0.5\,\mu g\,g^{-1}$ dry wt in R-calli but in trace amounts in G-calli. The differences in composition of the hydrodistillates from G-calli and from R-calli were not restricted to alkane hydrocarbons. The concentration of squalene in G-calli was more than six-fold greater than that of R-calli. Myristic and palmitic acids found in hydrodistillate from G-calli were absent from those of R-calli. Naphthalene, which was absent in G-calli, accumulated in R-calli at concentrations of $0.5\,\mu g\,g^{-1}$ dry wt (Table 2).

The lower concentration of squalene in R-calli may be due to higher rates of mobilization of this compound induced by higher concentrations of 2,4-D which is known to increase the sterol production in plant tissue cultures [24, 25]. Differences in composition of hydrodistillates of R-calli relatively to G-calli may, however, be correlated with the differentiation of abnormal root primordia. This may be the case of naphthalene produced by R-calli, although their functional correlation cannot be envisaged. Reports on the production of this compound by *in vitro* plant cultures are scarce. It was found in suspension cultures of *Solanum tuberosum* [26]. In *Magnolia* flowers, naphthalene is supposed to play a role in the attraction of insects to pollinate and, simultaneously, in protection of these plants against the insects attracted from feeding on floral parts [27].

According to some authors, two patterns of *n*-alkane distribution may be found in plant tissues [28], with the relative proportions depending on species. The Type A pattern has a Gaussian-like distribution of odd and even *n*-alkanes at equivalent amounts, mostly around C^{22}–C^{28}, produced by parenchymatic tissues, and the Type B pattern sharing an alternation in chain-length distribution, with the dominance of odd compounds (mostly C^{25}, C^{27}, C^{29}, C^{31} and C^{33}) produced by epidermis and located in the cuticular waxes. The *n*-alkanes identified in hydrodistillates of *O. v.* ssp. *virens* leaves correspond mostly to a type B pattern, indicating their probable origin in the cuticular wax. *n*-Alkanes produced by *O. v.* ssp. *virens* R-calli fit better with the type A pattern. However, in *O. v.* ssp. *virens* G-calli, tricosane, the only odd *n*-alkane produced in measurable amounts, represented one third of the total *n*-alkane amount, which was less than half of that produced by R-calli. The physiological function of *n*-alkanes in *in vitro* cultured plant tissues remains unclear. Odd and even *n*-alkane production by *in vitro* cultures of plant tissues and cells, has been reported ([25] and refs therein, [28, 29]). In photomixotrophic cell cultures of *Petroselinum crispum*, both type of *n*-alkanes were elicited by autoclaved fungal cells [30]. According to Weete *et al* [31]. regulation differences of the two hydrocarbon patterns can

be expressed in tissue cultures. Carriere *et al* [28]. showed that the type A pattern occurred in heterotrophic and photomixotrophic calli of *Euphorbia characias*, while the type B pattern occurred mainly in leaves and in photoautotrophic calli. The presence of fatty acids, namely palmitic acid, in hydrodistillates of G-calli and their absence in those of R-calli may be associated with differences in *n*-alkane composition. The greater ability of R-calli to decarboxylate fatty acids during chain elongation would explain the absence of fatty acids and the higher accumulation of odd carbon number *n*-alkanes in R-calli relative to G-calli.

EXPERIMENTAL

Plant material

Aerial parts of *O. vulgare* L. ssp. *virens* (Hoffmanns and Link) Ietswaart plants at the vegetative phase (May) were collected in the Natural Park of Peneda Gerês by the naturalists, Engª Helena and Engª Georgina. The material collected was used for calli induction and in the determination of essential oil composition. Calli induction was also tried from leaves collected in July. Voucher specimens (herbarium voucher number 183) are maintained in the herbarium of the National Park of Peneda Gerês.

Calli cultures

Leaves were immersed in 70% aq. ethanol for 2 min., surface-sterilised in a 10% NaOCl soln at 10% (v/v) for 10 min and rinsed $\times 3$ in sterile deionized H_2O. Sterilised mid-vein containing leaf segments of *ca* 1 cm^2 were laid on autoclaved MS [32] or B5 [33] basal medium supplemented with 2% sucrose, 1 mg l^{-1} 2,4-D and 1.0 or 0.5 mg l^{-1} BA and solidified with 0.8% agar after pH adjustment at 5.7. Solidified MS medium containing 2% sucrose and supplemented with 0.5 mg l^{-1} BA and 0.25 or 1.0 mg l^{-1} 2,4-D was used in the maintenance of *O. v.* ssp. *virens* calli performed through subcultures at one month intervals. Calli cultures were maintained at $25° \pm 2°$ under a 16/8 hr light/dark photoperiod with light radiation of *ca* 2000Lux provided by cool white fluorescent tubes (GE, 80W).

Hydrodistillation and analysis

Leaves (5 g fr. wt) of plants collected in May, were submitted to hydrodistillation in a Clevenger-type apparatus over 1 hr, using 1 ml of *n*-hexane for retention of hydrodistillate components. Hydrodistillation of calli in stationary phase of growth was performed following the same procedure. Known amounts of int. standard (*n*-heptadecane) were added to the *n*-hexane used to collect the hydrodistillate components of calli after confirmation of the absence of this compound in the cultures. All samples were analysed by GC and GC-MS. GC analysis were performed using a fused silica SGE BP-5 column (25 m long $\times 0.22$ mm ID $\times 0.25\ \mu$m film thickness of 5% phenyl dimethyl siloxane). Temp. programme: 50–280° at 5° min^{-1}, 14 min at 280°. Temp. 300° for injector and 320° for FID. H_2 was used as carrier gas with a column head pressure of 12 psi. GC–MS analysis was performed using a fused silica DB5 column (30 m long $\times 0.25$ ID, 0.25 μm film thickness composed of 5%-phenyl methylpolysiloxane) connected to an ion trap detector operating in EI mode at 70 eV. Injector and ion-source temps were 300° and 220°, respectively. The oven temp. program was as described above. He_2 was used as carrier gas with a column head pressure of 14 psi. Compounds were identified by comparing their MS with those in computer libraries or by comparison of their GC-retention times and MS with those of ref. compounds. The MS of compound 16 showed fragment ions at *m/z* (rel. int.) 137(34), 136[M-H_2O]$^+$ (100), 135(27), 121[M-H_2O-Me]$^+$ (99), 105(41), 93(8), 91(6), 81(8), 79(12) and 41(13), compatible with a terpineol isomer. The MS of compound 22: *m/z* (rel. int.) 189[M-Me]$^+$ (9), 161(23), 147(6), 136(8), 134(10), 133(8), 121(100), 119(34), 107(34), 105(32), 93(65), 91(52), 41(59), and the MS of compound 30: *m/z* (rel. int.) 204[M$^+$] (8), 189[M-Me]$^+$ (17), 161(21), 147(8), 136(17), 133(25), 121(100), 119(37), 107(45), 105(40), 93(60), 91(40), 41(53)) are both consistent with sesquiterpene hydrocarbons.

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