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BRIEF COMMUNICATION

Headspace-SPME of *in vitro* shoot-cultures and micropropagated plants of *Lavandula viridis*

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Abstract

In this work the volatiles emitted from *in vitro* shoot-cultures and micropropagated plants of *Lavandula viridis* L'Hér. were characterized and compared with those obtained from the field-grown mother-plant, using headspace solid phase micro-extraction following by capillary gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS). The headspace composition consisted mainly in oxygenated monoterpenes (66.7 - 79.2 %), where the major constituents emitted by the mature field-grown mother-plant, *in vitro* shoot-cultures and micropropagated plants were 1,8-cineole (74.0, 51.9 and 57.8 %) and camphor (2.9, 15.3 and 8.7 %), respectively. The headspace of *in vitro* shoot-cultures and micropropagated plants showed greater amount of α-pinene, camphene, β-pinene, β-selinene and selina-3,7(11)-diene, when compared with the field-grown mother-plant.

Additional key words: capillary GC/MS, 1,8-cineole, fragrances, lavender, micropropagation, volatiles.

The growing interest in plant secondary metabolites has intensified the development of efficient protocols for micropropagation of aromatic and medicinal species for phytochemical extraction (Tsuro *et al*. 2001, Bertoli *et al*. 2004, Ahuja *et al*. 2005). *Lavandula* species belong to the most popular ornamental and medicinal plants with great economic interest. *Lavandula viridis* L'Hér., commonly known as green or white lavender, is a xerophytic aromatic shrub endemic to southwest Iberian Peninsula. *In vitro* regeneration of *L. viridis* has been previously described (Dias *et al*. 2002) and the composition of the essential oils of this species has been also reported by our group, using conventional hydrodistillation techniques for isolation, followed by capillary gas chromatography coupled to mass spectrometry (GC/MS) analysis (Nogueira and Romano 2002). The volatile composition in the floral scent emitted *in vivo* by *L. viridis* plants had never been reported. Moreover, the knowledge of their pattern is important to identify the compounds that have relevance in the flavour profile. In recent years, several reports have used solid-phase micro-extraction (SPME), as a novel powerful analytical technique to characterize the chemical composition of the volatiles from several species (Palá-Paúl *et al*. 2004, Demirci *et al*. 2005). SPME offers several advantages over dynamic headspace or hydrodistillation techniques: it is less time consuming, a non-destructive, require smaller sample sizes, and minimise the formation of artefacts (Demirci *et al*. 2005, Flamini *et al*. 2005).

The aim of the present work was to investigate, for the first time, the chemical composition of the scent emitted by *in vitro* shoot-cultures and micropropagated plants of *L. viridis* in comparison with the field-grown mother-plant, using headspace solid phase micro-extraction followed by capillary gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS).

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Abbreviations: BA - benzyladenine; GC/MS - gas chromatography coupled to mass spectrometry; FP - field-grown mother plant; InV - *in vitro* shoot cultures; MP - micropropagated plants; MS - Murashige and Skoog medium; SPME - solid phase micro-extraction.
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In this work three types of plant material were used: *1*) *in vitro* shoot-cultures (InV) established from single node explants of the selected mature field-grown plant (Dias *et al*. 2002); *2*) shoots from micropropagated plants (MP) of the same clone, after acclimatization to *ex vitro* conditions; and *3*) shoots from a field-grown adult plant (FP) of *Lavandula viridis* L'Hér. collected at São Bartolomeu de Messines-Algarve, Portugal, selected for its high content of essential oils.

The procedures and conditions for establishment and multiplication *in vitro* of *L. viridis* were as described previously (Dias *et al*. 2002). In short, shoot-cultures were established from single node explants (10 - 12 mm long) from a mature field-grown mother-plant. These cultures were grown *in vitro* on Murashige and Skoog (1962; MS) medium supplemented with 0.44 μM 6-benzyladenine (BA). Shoots obtained at the end of establishment stage were multiplied on half-strength MS medium supplemented with 0.67 μM BA (InV). Shoots, 2 cm in length, harvested at the end of the multiplication stage, were rooted on ½ MS basal medium without growth regulators. All media were supplemented with 2 % sucrose and 7 % agar. The pH was adjusted to 5.8 prior to autoclaving for 20 min at 121 °C. Cultures were maintained at 25 ± 2 °C under a 16-h photoperiod provided by cool-white fluorescent tubes (*Philips* TLD 36W/33, 60 μmol m^{-2} s^{-1}). After potting and acclimatization, plantlets were transferred to the glasshouse and two months later they were used for volatiles analysis (MP).

For HS-SPME-GC/MS analysis, 0.5 g of fresh sample of each plant material (FP, InV and MP) was inserted separately into 30 cm^3 amber vials having screw caps (*Supelco,* Bellefonte, PA, USA). All the analyses were performed in triplicate. The SPME device (*Supelco*), coated with polydimethylsiloxane fibre (100 μm), was first activated by inserting it into the GC injector port at 250 ºC for 1 h. After the equilibration time, the fibre was exposed to the headspace for 15 min at room temperature (20 ºC). Following sampling, the SPME device was introduced into the injector port for chromatographic analysis and remained in the inlet for the run, thus preparing it to the next collection of volatiles. Blank assays using empty vials were conducted as controls.

Capillary GC/MS analyses were performed in an Agilent 6890 gas chromatograph interfaced to an *Agilent 5973 N* mass selective detector (*Agilent Technologies*, Palo Alto, USA). A vaporization injector was used in the splitless mode (2 min) at 250 ºC, with a fused silica capillary column, 30 m × 0.32 mm × 0.25 μm film thickness (HP-5MS; 5 % diphenyl 95 % dimethyl polydimethylsiloxane; *Agilent Technologies*). The oven temperature program was 40 ºC for 2 min and then increased at 5 ºC min^{-1} to 180 ºC, followed by 15 ºC min^{-1} to 240 ºC and held isothermally for 15 min. Helium was used as carrier gas with an average linear velocity of 30 cm s^{-1}.

Electron ionisation mass spectra in the range 40 - 400 Da were recorded at 70 eV. The quadrupole, source and transfer-line temperatures were maintained at 150, 230 and 280 ºC, respectively, and a turbo molecular pump (1.33 10^{-2} Pa) was used. All data were recorded using a *MS ChemStation* (*Agilent Technologies*, G1701CA; Rev C.00.01).

The identity of each compound was assigned by comparison of its retention index (RI), relative to C_9-C_{24} *n*-alkanes and according to literature (Adams 2001), as well as by comparison of the spectral data with the Wiley's library spectral data bank [G1035B;

Table 1. Constituents of the headspace from the field-grown mother-plant (FP), *in vitro* shoot-cultures (InV) and micropropagated plants (MP) of *Lavandula viridis* obtained by SPME-GC/MS ([a] - relative to C_9-C_{17} *n*-alkanes on the HP-5MS capillary column; [b] - normalized peak area abundances without using the correction factors.

Components	RI[a]	Composition[b] [%]		
		FP	InV	MP
Tricyclene	890	0.1	0.2	0.3
α-Pinene	902	0.3	8.0	8.3
Camphene	917	0.1	3.5	5.0
Sabinene	944	0.3	0.1	0.1
β-Pinene	947	0.1	2.7	2.6
Myrcene	963	0.1	0.5	-
Cymene	977	0.3	-	-
Δ^3-Carene	983	-	3.0	1.0
1,8-Cineole	1006	74.0	51.9	57.8
α-Terpinene	1037	-	0.1	-
trans-Linalool oxide	1038	0.4	-	-
Camphenilone	1050	0.1	-	-
cis-Linalool oxide	1061	0.4	-	-
α-Terpinolene	1069	-	0.3	-
6-Camphenone	1076	-	0.1	0.1
Camphor	1128	2.9	15.3	8.7
Δ-Terpineol	1151	0.4	-	-
Borneol	1152	-	0.2	-
cis-Pinocamphone	1160	0.3	0.1	-
4-Terpineol	1164	-	0.1	-
3-Oxocineole	1167	0.8	-	0.1
α-Terpineol	1177	-	0.2	-
Verbenone	1196	1.7	0.3	0.2
2-Oxocineole	1200	-	-	0.2
Bornyl acetate	1273	-	0.1	-
β-Elemene	1377	-	0.1	0.1
γ-Elemene	1414	-	0.5	0.5
β-Selinene	1464	0.3	1.4	2.1
trans-β-Guaiene	1491	-	0.8	1.1
Valencene	1506	-	1.3	1.5
Selina-3,7(11)-diene	1512	-	5.0	6.7
Monoterpene hydrocarbons		1.4	18.4	17.3
Oxygenated monoterpenes		79.2	67.9	66.7
Oxygenated sesquiterpenes		1.7	0.4	0.4
Sesquiterpene hydrocarbons		0.3	9.1	12.0
Total		82.6	95.8	96.4

Rev D.02.00] or homemade libraries (*Flavour 2.l*). For semi-quantification purposes, the normalized peaks area of each compound was used without any correction factor to establish abundance.

The volatiles emitted *in vivo* by *L. viridis* were shown to be complex mixtures of several components (Table 1). More than 30 compounds were positively identified, representing 82.6, 95.8 and 96.4 % of the total constituents of FP, InV and MP, respectively. The volatiles emitted were characterized by a high percentage of the monoterpene fraction, dominated by oxygenated monoterpenes (> 66.7 %). Although more compounds were identified in the essential oils of *L. viridis* isolated by Clevenger followed by capillary GC/MS analysis, HS-SPME-GC/MS confirmed the monoterpenes predominance previously observed (Nogueira and Romano 2002). The volatiles emitted from *L. viridis* FP, InV and MP, showed higher abundance of oxygenated monoterpenes (79.2, 67.9 and 66.9 %, respectively) and monoterpene hydrocarbons (1.4, 18.4 and 17.3 %), and lower amounts of sesquiterpene hydrocarbons (0.3, 9.1 and 12.0 %) and oxygenated sesquiterpenes (1.7, 0.4 and 0.4 %) (Table 1).

The different plant materials analysed in the present study showed qualitative differences in the composition of the volatiles emitted. InV and MP showed higher content of monoterpene hydrocarbons (18.4 and 17.3 %) and sesquiterpene hydrocarbons (9.1 and 12.0 %) than FP, while the amount of oxygenated monoterpenes (67.9 and 66.7 %) and oxygenated sesquiterpenes (0.4 and 0.4 %) were lower (Table 1). The most important volatile constituent emitted by the three types of plant material studied is 1,8-cineole (51.9 - 74.0 %), confirming previous results reported from the essential oils of *L. viridis* (Nogueira and Romano 2002) and results with other *Lavandula* species (Barocelli *et al*. 2004, Ballabeni *et al*. 2004, Sanz *et al*. 2004). This compound gives the particular flavour characteristic of the lavender fields and, as expected, a remarkable abundance occurred in the headspace vapour, while in the oil extracted from *L. viridis* lower amounts were found (18.2 - 25.1 %) (Nogueira and Romano 2002). This could be due to differences between headspace-SPME technique and conventional hydrodistillation techniques used to isolate the essential oil. It must be emphasize that 1,8-cineole is an important fragrance compound, used in practice.

Regarding the other main volatile constituents (> 5 %), some important compositional differences could be observed between the three types of plant material.The headspace of the InV and MP showed higher percentages of camphor (15.3 and 8.7 %, respectively), α-pinene (8.0 and 8.3 %), camphene (3.5 and 5.0 %), β-pinene (2.7 and 2.6 %) and β-selinene (1.4 and 2.1 %) than FP, whereas FP had higher percentage of verbenone (1.7 %). Δ^3-Carene, valencene and selina-3,7(11)-diene occurred in InV and MP and were not detected in FP. Other minor compounds (< 0.5 %), such as cymene, camphenilone, *trans*-linalool oxide and *cis*-linalool oxide found in FP, were not detected in InV and MP (Table 1).

Some studies reported from other species (Arikat *et al*. 2004) and *Lavandula* species in particular (Tsuro *et al*. 2001), demonstrated that the chemical composition of the compounds detected in *in vitro* cultures could be higher than those detected in field-grown plants. Under *in vitro* conditions, harvested shoots are young, and thus the amount of chemical compounds that are secreted by the trichomes is more concentrated. In addition, the increase of some compounds in InV and MP may be related to the influence of BA in the culture medium that had a positive effect on the capacity of *Lavandula dentata* plantlets to produce and/or accumulate essential oils (Sudria *et al*. 1999, 2001). The highest amount of camphor in the InV (15.3 %) when compared to MP (8.7 %) and particularly in the FP (2.9 %), may be also related to the effect of BA in the culture medium as showed Tawfik *et al*. (1992) in *Salvia officinalis*.

In conclusion, HS-SPME-GC/MS methodology gave us the possibility to investigate the volatiles emitted directly from fresh plants of *L. viridis* and to define its chemical composition. Volatiles from *in vitro* shoot cultures (InV) or micropropagated plants (MP), when compared with those obtained from the field-grown mother-plant (FP), present the same major component (1,8-cineole) without remarkable quantitative variation. Additionally, oxygenated monoterpenes were found in considerable higher amount in all three types of plant material, and monoterpene hydrocarbons particularly in InV and MP. Some of these compounds have industrial and medical applications, therefore, micropropagation under controlled conditions could be an interesting alternative for field production of *L. viridis*.

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