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Essential oils from hairy root cultures and from plant roots of *Achillea millefolium*

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Abstract

The essential oils isolated from roots of two *Achillea millefolium* populations (BGL and CGA) and from two hairy root cultures (A4 and LBA) derived from one of these were analysed by GC and GC–mass spectrometry. The essential oils from the plant roots were obtained in a yield of 0.10% (BGL) and 0.05% (CGA) (v/w), whereas that of both hairy root cultures attained 0.05% (v/w). Compared on a dry weight basis, the yield from the hairy root cultures was similar to or higher than that from the plant roots. The oxygen-containing sesquiterpene fraction of the root oils from both plant populations was the most characteristic one, *epi*-cubenol being the main component of these oils (18% for BGL and 26% for CGA). Oxygen-containing monoterpenes constituted the most important fraction of the essential oils from both hairy root cultures, neryl isovalerate being the main component (27% and 43% for A4 and LBA, respectively).

No major differences were detected in the root essential oil profiles of the two *A. millefolium* plant populations, but qualitative and quantitative differences were found between the essential oils from the plant roots and those from the hairy roots, and also between the oils from the two hairy root cultures.

Keywords: *Achillea millefolium*; Asteraceae; *Agrobacterium rhizogenes*; Hairy root cultures; Plant roots; Essential oil

1. Introduction

The composition of the essential oil isolated from aerial parts of *Achillea millefolium* (Asteraceae) has been a subject of extensive studies (Falk, Bauer, Bell, & Smolenski, 1974; Haggag, Shalaby, & Verzar-Petri, 1975; Bélanger, Dextraze, Lachance, & Savard, 1991; Chatzopoulou, Katsiotis, & Baerheim Svendsen, 1992; Figueiredo, Barroso, Pais, & Scheffer, 1992; Hofmann, Fritz, Nitz, Kollmannberger, & Drawert, 1992; Bélanger & Dextraze, 1993; Afsharypuor, Asgary, & Lockwood, 1996), mainly due to its medicinal properties (Font Quer, 1981; Chandler, Hooper, & Harvey, 1982; Gadgoli & Mishra, 1995) and taxonomic value (Eglseer, Jurenitsch, Saukel, Franz, & Kubelka, 1988). The roots have been neglected as a source of essential oil, although yarrow, like many other members of the Asteraceae, possesses not only trichomes (Stahl, 1953; Figueiredo & Pais, 1994), but also oil ducts, that can also be found in the plant roots.

The essential oil production by *A. millefolium* cell suspension cultures has also been studied, but although these cultures are able to produce an essential oil with components not found in the parent plant (Figueiredo, Pais, & Scheffer, 1995) and show the ability to biotransform terpenes (Figueiredo, Almendra, Barroso, & Scheffer, 1996), the major constraint is the low oil

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Table 1
Percentage composition of the essential oils of *Achillea millefolium* isolated from roots of two plant populations (Botanical Garden of Lisbon is BGL (parent plant roots) and Canecão Garden of Almada is GCA) and from two hairy root cultures (A4 and LBA) grown in MS/2 (half strength MS medium) under dark conditions. t means trace (< 0.05%)

Components	R.I.[a]	Plant roots		Hairy roots	
		BGL	CGA	A4	LBA
α-Thujene	924		0.1	t	t
α-Pinene	930	0.6	1.3	t	t
Camphene	938	t	0.2		t
Sabinene	958		0.7		
β-Pinene	963	4.4	6.5		0.1
β-Myrcene	975	0.1	0.4		0.1
α-Phellandrene	995		0.1		
α-Terpinene	1002		0.3		
Phenylacetaldehyde	1002		t		
p-Cymene	1003		0.4		
1,8-Cineole	1005	t	1.5		
Limonene	1009	0.1	1.5	0.3	0.1
cis-β-Ocimene	1017	t	t		t
trans-β-Ocimene	1027	t	t		0.1
γ-Terpinene	1035		0.2		
trans-Sabinene hydrate	1037		t		
Terpinolene	1064	t	0.3		0.2
Linalool	1074	0.1	0.2	t	0.5
Camphor	1095		2.0		
allo-Ocimene	1110	0.1			
trans-Verbenol	1114	t	t		
Decenal[b]	1116			1.1	0.4
Terpinen-4-ol	1148	t	0.7		
α-Terpineol	1159	0.4	0.3	0.7	1.2
Nerol	1206	t		6.7	5.2
Geranial	1240			0.6	0.3
Eugenol	1327				t
δ-Elemene	1332	6.9	3.6	1.0	2.1
Chrysanthenone	1361		0.3		
α-Copaene	1375			0.1	t
β-Maaliene[b]	1375				0.1
Isocomene[b]	1375	0.5	t	0.4	0.5
β-Elemene	1388	1.0	0.7	1.1	0.2
β-Isocomene[b]	1388			0.4	0.2
β-Caryophyllene	1414	0.7	0.9	1.4	0.7
α-Santalene	1434	0.1	0.1	t	
trans-α-Bergamotene	1434	0.1	t	0.3	0.1
β-Santalene[b]	1435	1.8	t	0.6	
α-Humulene	1447	t	0.3	t	0.2
trans-β-Farnesene	1456	3.2	2.2	10.0	7.6
Allo-Aromadendrene	1456				t
γ-Himachalene	1468	0.7		0.9	2.2
Germacrene-D	1474	2.0	2.0	0.4	
cis-β-Farnesene	1476			1.9	1.1
Bicyclogermacrene	1487			0.9	
α-Muurolene	1494			0.7	
β-Bisabolene	1495			0.4	
γ-Cadinene	1496	0.8	0.6	0.4	
trans,*trans*-α-Farnesene	1500			1.5	0.8
δ-Cadinene	1505	1.5	1.2	2.7	0.4
Sesquiphellandrene	1508	2.9	0.7	2.0	0.4
Germacrene-D-4-ol	1557	4.5			
Neryl 2-methylbutyrate	1564			2.9	2.8
Neryl isovalerate	1570	1.0	0.5	27.4	42.7
Guaiol	1575	0.7	t		
Unidentified A	1584			7.1	1.1
epi-Cubenol	1600	18.0	26.1		17.4
epi-Guaiol	1600	t	t		

Table 1 (*continued*)

Components	R.I.[a]	Plant roots		Hairy roots	
		BGL	CGA	A4	LBA
T-Cadinol	1616	2.9	3.9	0.9	
δ-Cadinol	1618			0.9	
α-Cadinol	1626	4.2	2.9	3.4	
Identified		59.3	62.7	72.0	87.7
Grouped components					
Monoterpene hydrocarbons		5.3	12.0	0.3	0.6
Oxygen-containing monoterpenes		1.5	5.5	38.3	52.7
Sesquiterpene hydrocarbons		22.2	12.3	27.1	16.6
Oxygen-containing sesquiterpenes		30.3	32.9	5.2	17.4
Others				1.1	0.4
Yield (v/fr.wt.)		0.10	0.05	0.05	0.05
Yield (v/dry wt.)		0.71	0.36	0.75	0.75

[a] Relative to C_9–C_{17} *n*-alkanes on a DB-1 column.
[b] Based on mass spectra only.

yield attained (Figueiredo et al., 1995). This low yield is a general drawback of most undifferentiated cell cultures (Deans & Svoboda, 1993).

Fast growing adventitious transgenic roots obtained by infection of dicotyledonous plants with *Agrobacterium rhizogenes* can be viewed as a way of establishing 'immortal' cell lines which can be useful in plant tissue culture. In this process, bacterial genetic material is integrated in the plant chromosome causing the proliferation of tumorous roots. Hairy root cultures are potentially applicable to the production of root-derived metabolites. An important part of the research concerning hairy root secondary metabolites is related to the production of medicinally important alkaloids, though many other metabolites have been investigated such as thiophenes, peroxidases, essential oils and saponins (Deans & Kennedy, 1991). A previous attempt to establish *A. millefolium* hairy root cultures was not successful because poor root development prevented multiplication of clonal material (Mugnier, 1988).

As part of a program to study the potentialities of hairy root cultures for essential oil production, we report in this paper on a comparison between the essential oils from the roots of two plant populations and those from two hairy root cultures of *A. millefolium* derived from the BGL plant population.

2. Results and discussion

The yellowish essential oil isolated from the plant roots was obtained in a yield of 0.10 and 0.05% (v/w) for the Botanical Garden of Lisbon (BGL) and the Canecão Garden of Almada (CGA) populations, respectively. Thirty-six components of the oil from the BGL roots were identified, amounting to 59% of the essential oil (Table 1). Oxygen-containing sesquiterpenes constituted the major fraction of the oil, attaining 30%, *epi*-cubenol (18%) being its main component. Another important fraction was that of the sesquiterpene hydrocarbons (22%), from which δ-elemene (7%) was the major component. The monoterpene fraction amounted only to 7%, β-pinene (4%) being its main constituent. Forty-two components were identified in the oil isolated from the CGA roots, amounting to 63% of the total oil Table 1. The main component, again *epi*-cubenol (26%), represented a significant part of the major fraction of the oil, i.e. that of the oxygen-containing sesquiterpenes (33%). The sesquiterpene hydrocarbons (12%) were again dominated by δ-elemene (4%). The monoterpene fraction (18%) was present in a larger amount than that found for the BGL population, but β-pinene (7%) was again the major constituent of this fraction. About 40% of both root oils could not be identified until now; these parts of the oils consisted of one or two components that amounted to 5%, another one or two were about 3%, and the remaining constituents were less than 1%.

In general, the root oils from the two plant populations revealed some qualitative similarities, at least with regard to their main components. Nevertheless, the oil isolated from the CGA population was richer in several minor monoterpenes, whereas germacrene-D-4-ol (5%) present in the oil from the BGL population could not be found in that from the CGA. The composition of these root oils was different from that of the corresponding aerial parts (Figueiredo, Barroso, Pais, & Scheffer, 1992). According to Figueiredo et al.

(1992), the aerial parts of the same plant populations also showed some differences in essential oil composition. Although the monoterpene fraction was dominant in the oils from the aerial parts of both populations, the main components differed: sabinene and 1,8-cineole were dominant in the oil isolated from the plants of the BGL, whereas camphor, 1,8-cineole and β-pinene were the main constituents of the oil from the CGA plants.

The yellowish essential oils isolated from the two hairy root cultures (derived from the BGL plant population) were obtained in a yield of 0.05% (v/w). When expressed on a fresh weight basis, the oil yield of the two hairy root cultures was similar to that of the CGA plant roots, and half of that obtained for the BGL parent plant roots Table 1. However, calculated on a dry weight basis, the oil yield of the hairy roots was similar to that obtained for the BGL parent plant roots and higher than that of the CGA plant roots, because of the higher water content of the hairy roots (1 g fr. wt. = 66 mg dry wt.) compared with that of the plant roots (1 g fr. wt. = 140 mg dry wt.). Similar results were obtained by Santos et al. (1998), when comparing the oil yield of anise hairy roots with those of the fruits and roots of the parent plant.

The essential oil yield obtained for the *A. millefolium* hairy roots (0.05%) was 50 times higher than that reported for yarrow cell suspension cultures (0.001%) (Figueiredo et al., 1995), and it is not surprising that also their oil composition was completely different, considering their distinctly different nature and culture conditions.

Thirty-four components of the oil isolated from the A4 hairy root culture were identified, amounting to 72% of the total oil Table 1. The fraction of oxygen-containing monoterpenes was dominant in this oil (38%), neryl isovalerate (27%) and nerol (7%) being the main components. Sesquiterpene hydrocarbons constituted the second major fraction of the oil (27%), *trans*-β-farnesene (10%) being its main constituent. Present in a relatively large amount (7%) was an unidentified component (A), the mass spectral data of which are given in Section 3.

Also thirty-four components of the oil isolated from the LBA hairy root culture were identified, amounting to 88% of the total oil Table 1. The oxygen-containing monoterpene fraction of the oil (53%) was again the most abundant one, neryl isovalerate (43%) and nerol (5%) being again the main components. Sesquiterpene hydrocarbons and oxygen-containing sesquiterpenes were present in similar amounts (17%), the main components being *trans*-β-farnesene (8%) and *epi*-cubenol (17%), respectively. Compound A attained only 1% in this oil.

The main difference between the oils from the two hairy root cultures was due to the presence of *epi*-cubenol (17%) in the LBA clone, whereas it was not detected in the A4 clone. This compound is the main component of both plant root oils. The unidentified component A was found in a much smaller amount in the oil from the LBA culture than in that from the A4 culture. It is noteworthy that preliminary studies (Lourenço, 1998) showed only some minor differences in the composition of the two hairy root oils; about one year after initiation of the cultures a stabilization in the oil composition had occurred, and the clear differences we describe in the present paper were observed.

Although the differences found in the essential oil composition of both hairy root cultures could be imputed directly to the fact that they were induced by different *A. rhizogenes* strains, as reported for other plant species (Hu & Alfermann, 1993; Oksman-Caldentey & Hiltunen, 1996; Yu, Kwok, & Doran, 1996), it should be kept in mind that not only a different degree of bacterial DNA integration may occur, but also that the individuals of a plant species are usually not genetically uniform. Thus, even when transformed with the same *A. rhizogenes* strain, different results, in terms of growth and metabolite production, can be achieved. In spite of the fact that transgenic roots show a high genotypic and phenotypic stability, when compared with undifferentiated cell cultures, some differences can be found between individual clones. According to Yu et al. (1996) hairy root clones established from different root meristems of the same culture can show different growth rates along with differences in metabolite production.

Comparative studies on the essential oils from parent plant roots and from hairy roots of *Valeriana officinalis* (Gränicher, Christen, & Kapetanidis, 1992) showed also numerous differences between the two oil profiles. According to Kennedy, Deans, Svoboda, Gray, and Waterman (1993) the essential oil composition of *Artemisia absinthium* parent plant roots and that of hairy roots were completely different. According to these authors, the maturity of the root system may be important for the essential oil synthesis. The continuous growth characteristic of transgenic roots may not be compatible with high degrees of maturation and the metabolite production may mirror this (Payne, Bringi, Prince, & Shuler, 1991). Moreover, the role of metabolites translocation along the plant system must not be underestimated. The fact that hairy root culture systems are constituted by isolated roots may deprive the system of precursors and other compounds with an origin elsewhere in the parent plant. Additionally, compounds that in an in vivo system would be translocated into other plant organs, may remain in the place of synthesis or may be excreted to the culture medium, when an in vitro system is considered. The conditioning effect of environ-

mental factors in vivo may determine some metabolic differences as well.

3. Experimental

3.1. Plant material

A. millefolium roots were collected from plants growing in the Botanical Garden of Lisbon (BGL) and in the Canecão Garden of Almada (CGA), in May 1996. Voucher specimens have been deposited in the Herbarium of the Instituto Botânico da Faculdade de Ciências de Lisboa (LISU: 160056 and 160098, respectively). Yarrow seedlings used in the transformation procedure described below were grown under aseptic conditions from seeds collected from the BGL plant population in September 1995. The seeds were soaked overnight in H_2O prior to washing in a 10% detergent solution for 10 min, followed by surface disinfection with 70% EtOH for 30 s and 6% NaOCl for 5 min. After rinsing four times with sterile H_2O, the seeds were germinated on solid Schulz medium (Schulz, 1981), without growth regulators, at 24°C in a 16 h light/8 h dark photoperiod (37 $\mu Em^{-2}\ s^{-1}$).

3.2. Bacterial strains

Strains of *A. rhizogenes* (LBA 9402 and A470GUS, respectively) were maintained on solid YMB medium (Hooykaas, Klapwijk, Nuti, Schilperoort, & Rorsch, 1977). Each strain was inoculated in liquid YMB medium, 48 h prior to transformation assays.

3.3. Transformation procedure

Two-week-old *A. millefolium* seedlings were randomly wounded with the tip of a sterile hypodermic needle carrying one drop of the bacterial suspension. The seedlings were cocultivated with the bacteria on MS/2 solid medium (half strength Murashige and Skoog medium (Murashige & Skoog, 1962)), with 30 g/l of sucrose, in a 16 h light/8 h dark photoperiod at 24°C, for 48 h. They were then transferred to solid MS/2 medium supplemented with antibiotics (500 mg/l ampicillin or 250 mg/l carbenicillin plus 250 mg/l cefotaxime). Roots that developed were transferred to liquid MS/2 medium without antibiotics, maintained in the dark at 24°C on an orbital shaker (80 rpm) and subcultured every 15 days.

3.4. Dry weight determination

Root samples from the two plant populations, and filtered hairy roots from both cultures, were freeze-dried for 2 days, at 10^{-1} mbar and −42°C.

3.5. Isolation procedure

The essential oils, both from hairy root cultures and from roots of both plant populations, were isolated by distillation–extraction for 3 h, using a Likens–Nickerson-type apparatus (Likens & Nickerson, 1964) with *n*-pentane as solvent, and by hydrodistillation for 3 h, using a Clevenger-type apparatus (Anonymous, 1975). The oil samples isolated by hydrodistillation were used to estimate the oil yields, and those isolated by distillation–extraction to determine the percentage composition of the oils, since the chance of artefact formation must be considered smaller when the latter method is used. For each of the two hairy root cultures, the material used for essential oil isolation was collected periodically, i.e. about 10 times over a period of about one year after stabilization of cultures had occurred; in all cases the hairy roots were harvested on the 15th day after subculturing.

3.6. Synthesis of neryl isovalerate and neryl 2-methylbutyrate

The nerol esters were prepared from 2-methylbutyric acid chloride or isovaleric acid chloride in equimolar amounts in pyridine. The reaction took place at 60°C for 1 h.

3.7. Gas chromatography

GC analyses were performed using a twin FID instrument, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific Inc) and a DB-Wax fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific Inc). The oven temp. was programmed, 45–175°C, at 3°C min^{-1}, subsequently at 15°C min^{-1} up to 240°C and then held isothermal for 10 min; injector and detector temps were 220°C and 240°C, respectively; carrier gas, H_2 at 30 cms^{-1}. Samples were injected using the split-sampling technique with a ratio of 1:50. Percentage composition of oils was computed using the normalization method from the GC peak areas without correction factors. Percentage data shown are mean values of two injections of each of the oils from the plant material; for the ca. 10 oil samples isolated from the hairy root material harvested periodically, mean values of all of these samples were calculated after each sample was injected twice.

3.8. Gas chromatography–mass spectrometry

The GC–MS unit was equipped with a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25

μm; J&W Scientific Inc) and interfaced with an ion trap detector (ITD; software version 4.1). Oven temps were as described above; transfer line temp., 280°C; ion trap temp., 220°C; carrier gas, He at 30 cms^{-1}; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their RIs, relative to C_9–C_{17} *n*-alkanes and MS with corresponding data of components of reference oils or of synthetic compounds.

3.9. MS data from the unidentified compound A

m/z (%) = 172 (82), 157 (13), 144 (37), 141 (32), 129 (23), 115 (46), 113 (38), 112 (17), 111 (22), 110 (29), 109 (14), 101 (97), 88 (17), 87 (100), 86 (69), 85 (21), 75 (30), 74 (17), 65 (56), 62 (21), 61 (21), 55 (21), 50 (11).

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References

Afsharypuor, S., Asgary, S., & Lockwood, G. B. (1996). *Flavour Fragr. J.*, *11*, 265.

Anonymous (1975). In (p. 68). In *European pharmacopoeia*, *Vol. 3*. Sainte-Ruffine: Maisonneuve SA.

Bélanger, A., & Dextraze, L. (1993). *Acta Hortic.*, *330*, 141.

Bélanger, A., Dextraze, L., Lachance, Y., & Savard, S. (1991). *22th International Symposium on Essential Oils, St. Vincent, Aosta, Italy, 1991* abstract.

Chandler, R. F., Hooper, S. N., & Harvey, M. J. (1982). *Econ. Bot.*, *36*, 203.

Chatzopoulou, P., Katsiotis, S. T., & Baerheim Svendsen, A. (1992). *J. Essent. Oil Res.*, *4*, 457.

Deans, S. G., & Kennedy, A. I. (1991). *Agro. Industry Hi-Tech.*, *6*, 11.

Deans, S. G., & Svoboda, K. P. (1993). In R. Hay, & P. Waterman, *Volatile oil crops* (pp. 113–136). Essex: Longman Scientific and Technical.

Eglseer, K., Jurenitsch, J., Saukel, J., Franz, Ch., & Kubelka, W. (1988). *Sci. Pharm.*, *56*, 15.

Falk, A. J., Bauer, L., Bell, C. L., & Smolenski, S. J. (1974). *Lloydia*, *37*, 598.

Figueiredo, A. C., Almendra, M. J., Barroso, J. G., & Scheffer, J. J. C. (1996). *Biotechnol. Lett.*, *18*, 863.

Figueiredo, A. C., Barroso, J. G., Pais, M. S. S., & Scheffer, J. J. C. (1992). *Flavour Frag. J.*, *7*, 219.

Figueiredo, A. C., Barroso, J. G., Pais, M. S. S., & Scheffer, J. J. C. (1992). *J. Chromatogr. Sci.*, *30*, 392.

Figueiredo, A. C., & Pais, M. S. S. (1994). *Ann. Bot.*, *74*, 179.

Figueiredo, A. C., Pais, M. S. S., & Scheffer, J. J. C. (1995). *Plant Cell Tiss. Org. Cult.*, *40*, 113.

Font Quer, P. (1981). In *Plantas medicinales, el dioscórides renovado* (p. 803). Barcelona: Editorial Labor SA.

Gadgoli, C., & Mishra, S. H. (1995). *Fitoterapia*, *66*, 319.

Gränicher, F., Christen, P., & Kapetanidis, I. (1992). *Plant Cell Rep.*, *11*, 339.

Haggag, M. Y., Shalaby, A. S., & Verzar-Petri, G. (1975). *Planta Med.*, *27*, 361.

Hofmann, L., Fritz, D., Nitz, S., Kollmannberger, H., & Drawert, F. (1992). *Phytochemistry*, *31*, 537.

Hooykaas, P. J. J., Klapwijk, M., Nuti, M. P., Schilperoort, R. A., & Rorsch, A. (1977). *J. Gen. Microbiol.*, *98*, 477.

Hu, Z. B., & Alfermann, A. W. (1993). *Phytochemistry*, *32*, 699.

Kennedy, A. I., Deans, S. G., Svoboda, K. P., Gray, A. I., & Waterman, P. G. (1993). *Phytochemistry*, *32*, 1449.

Likens, S. T., & Nickerson, G. B. (1964). *Am. Soc. Brew. Chem. Proc*, 5.

Lourenço, P. M. L. (1998). Master thesis, Faculty of Sciences of Lisbon, Lisbon.

Mugnier, J. (1988). *Plant Cell Rep.*, *7*, 9.

Murashige, T., & Skoog, F. (1962). *Physiol. Plant.*, *15*, 473.

Oksman-Caldentey, K., & Hiltunen, R. (1996). *Field Crops Res.*, *45*, 57.

Payne, G. F., Bringi, V., Prince, C. L., & Shuler, M. L. (1991). In *Plant cell and tissue culture in liquid systems* (pp. 225–279). Munich: Hanser Publishers.

Santos, P. M., Figueiredo, A. C., Oliveira, M. M., Barroso, J. G., Pedro, L. G., Deans, S. G., Younus, A. K. M., & Scheffer, J. J. C. (1998). *Phytochemistry*, *48*, 455.

Schulz, A. (1981). Ph.D. thesis, Technische Universität, Hannover.

Stahl, E. (1953). *Z. Bot.*, *41*, 123.

Yu, S., Kwok, K., & Doran, P. (1996). *Enzyme Microbial Technol.*, *18*, 238.