Pergamon

PII: S0031-9422(98)00022-3

Phytochemistry, Vol. 48, No. 3, pp. 455–460, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

ESSENTIAL OILS FROM HAIRY ROOT CULTURES AND FROM FRUITS AND ROOTS OF *PIMPINELLA ANISUM*

PAULA M. SANTOS, A. CRISTINA FIGUEIREDO,* M. MARGARIDA OLIVEIRA, JOSÉ G. BARROSO, LUIS G. PEDRO, STANLEY G. DEANS,† A. K. M. YOUNUS‡ and JOHANNES J. C. SCHEFFER‡

Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1780 Lisbon, Portugal; † Department of Biochemical Sciences, SAC Auchincruive, Ayr KA6 5HW, U.K.; ‡ Division of Pharmacognosy, LACDR, Leiden University, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands

(*Received in revised form* 1 *December* 1997)

Key Word Index—*Pimpinella anisum*; Apiaceae; anise; essential oil; hairy root cultures; fruits; roots; pseudoisoeugenols; C_{12} compounds.

Abstract—Hairy root cultures of *Pimpinella anisum* were established following inoculation of aseptically grown plantlets with an A_4 pRiA_4 70 GUS strain of *Agrobacterium rhizogenes*. The essential oils from the hairy roots, maintained in four different media, and from the fruits and roots of the parent plant were analysed and their compositions compared by GC and GC-mass spectrometry. The major components of the essential oils from the hairy root cultures were *trans*-epoxypseudoisoeugenyl 2-methylbutyrate, geijerene, pregeijerene, zingiberene and β-bisabolene, in varying amounts depending on the light or dark growth conditions and on the culture media tested. *trans*-Epoxypseudoisoeugenyl 2-methylbutyrate, β-bisabolene and pregeijerene were the major components of the essential oil from the roots of the parent plant, whereas the main component of the fruit oil was *trans*-anethole. Geijerenes were not detected in the fruit oil. The essential oil yield of the transformed roots grown in one of the media was comparable with that obtained for the roots of the parent plant and, calculated on a dry weight basis, the oil yield of these hairy roots was comparable with that of the fruits.

INTRODUCTION

Pimpinella anisum, anise, is a herbaceous annual plant, native to the Mediterranean region and Egypt. Anise is primarily grown for its fruits, commercially called "seeds", that are currently used for flavouring, but the essential oil from the fruits is also valuable in perfumery and in medicine [1, 2].

Considering the enhanced biosynthetic capacity of transformed root cultures when compared with that of unorganized cell suspension cultures, the aim of this work was to establish *P. anisum* transformed root cultures and to compare their essential oil production under different growth conditions with that of the fruits and roots of the parent plant.

RESULTS AND DISCUSSION

Fast growing anise hairy root cultures were established on solid MS/2 medium, by transformation with an A_4 strain of *Agrobacterium rhizogenes*. Hairy root cultures were then initiated in four hormone-free liquid culture media (B5, B5/2, MS/2 and SH). Although the hairy roots grown in the different media showed initially a similar density of root hairs and a similar branching ability, they gradually diversified and, after some time, the hairy roots grown in MS/2 and B5 media in darkness, showed a remarkable tendency to develop callus tissue.

With respect to their growth rate, from the four media tested, the hairy root cultures grown in SH medium in darkness showed the highest rate (Fig. 1). Hairy root cultures grown in MS/2 medium, which under photoperiod conditions also showed a high growth rate, had the lowest rate under dark conditions. Hairy root cultures grown in the B5 and B5/2 media showed the lowest growth rate under photoperiod conditions and a marked increase in growth when grown in darkness.

The identified components of the oils isolated from the hairy roots and from the fruits and roots of the parent plant are listed, together with their percentages, in order of their elution from a DB-1 column in Table 1. The essential oils isolated from hairy roots grown in different culture media were qualitatively similar but showed large quantitative differences regarding the major components. In the cultures grown in the

* Author to whom correspondence should be addressed.

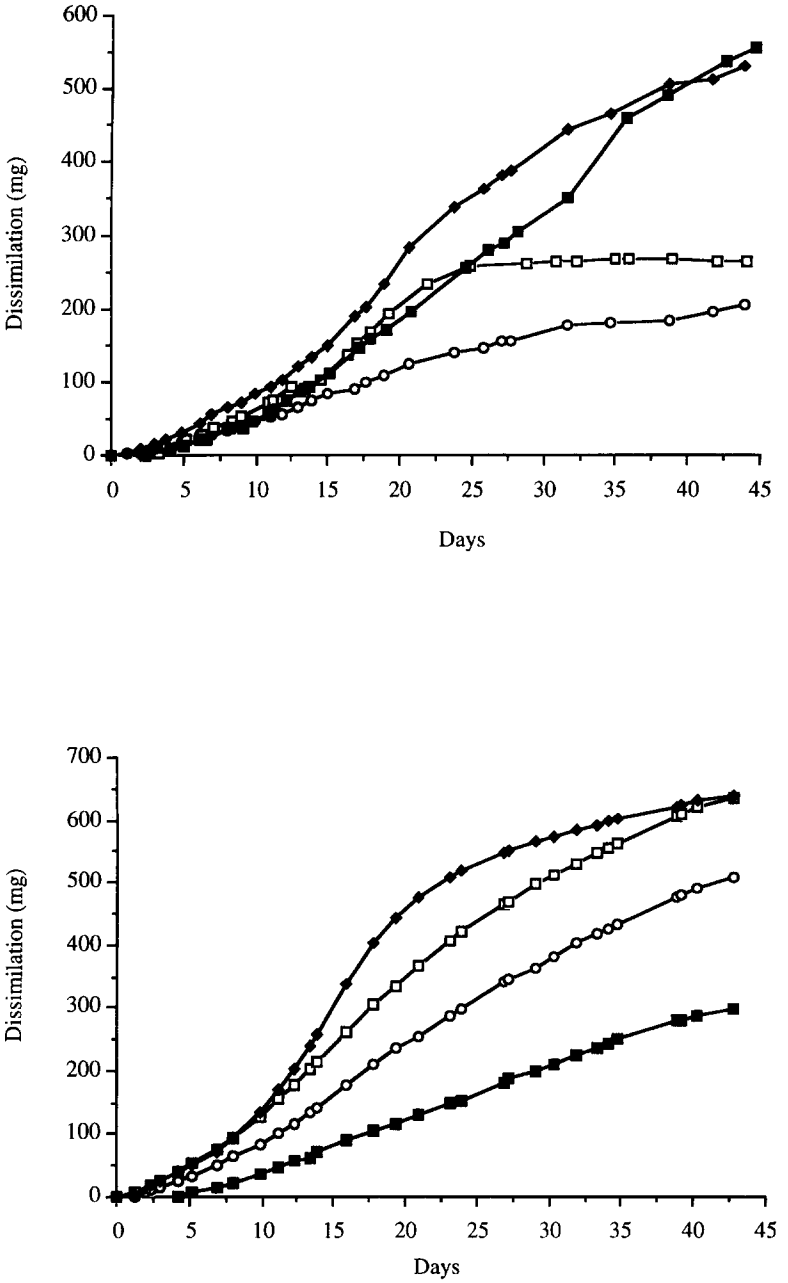

Fig. 1. Dissimilation growth curves obtained for hairy roots of *P. anisum* grown in B5 (□), B5/2 (○), MS/2 (■) and SH (◆) media, under photoperiod (upper graph) or dark (lower graph) conditions.

dark, the main component was always *trans*-epoxypseudoisoeugenyl 2-methylbutyrate, whereas in those grown under photoperiod conditions, the main component was either this compound (B5/2) or pregeijerene (B5, MS/2 and SH) (Table 1). The occurrence of rare phenylpropanoids from the pseudoisoeugenol group, such as *trans*-epoxypseudoisoeugenyl 2-methylbutyrate, was first detected in the fruit essential oil of *P. anisum* [3] and, since then, it has been detected only in *Pimpinella* species, both in plants and *in vitro*, namely in shoot and callus cultures [4–8]. Geijerenes have also been detected previously, not only in species of the Apiaceae [9], but also in members of the Asteraceae, Lamiaceae, Rosaceae and Rutaceae [10–18] (Table 2).

Major differences, both qualitative and quantitative, were found between the essential oils isolated from the hairy roots and the oil isolated from the fruits. *trans*-Anethole, clearly the main component of the fruit oil, was found only in traces or in amounts of ca 1%, in the oils from hairy roots (Table 1). Conversely, the major components of hairy root oils, namely *trans*-epoxypseudoisoeugenyl 2-methylbutyrate, geijerene, pregeijerene, zingiberene and β-

Table 1. Percentage composition of essential oils of *Pimpinella anisum* isolated from the fruits, roots and from hairy roots grown in B5/2, MS/2 (B5/2 and MS/2 = half strength media), SH and B5 media under photoperiod or dark conditions

				B5/2		MS/2		SH		B5	
Components	R.I.*	Fruits	Roots	Light	Dark	Light	Dark	Light	Dark	Light	Dark
n-Heptanal	897	—	0.2	t	t	t	t	t	t	t	t
Benzaldehyde	927	—	0.7	—	—	—	—	—	—	—	—
Sabinene	958	—	0.2	—	—	—	—	—	—	—	—
n-Octanal	973	—	0.1	0.4	1.3	3.0	2.3	1.7	1.4	3.3	0.2
Myrcene	975	—	0.1	—	—	—	—	—	—	—	—
Decane	1000	—	—	t	t	t	t	t	t	t	t
α-Terpinene	1002	—	t	—	—	—	—	—	—	—	—
Phenylacetaldehyde	1002	—	—	t	t	t	t	t	t	t	t
p-Cymene	1003	—	t	t	t	t	t	t	t	t	t
Limonene	1009	—	t	t	t	t	t	t	t	t	t
γ-Terpinene	1035	—	0.1	—	—	—	—	—	—	—	—
m-Cresol	1048	—	—	0.2	t	1.5	t	0.4	0.2	0.5	t
n-Nonanal	1073	—	—	t	t	t	t	t	t	t	t
Undecane	1100	—	t	0.3	0.2	2.8	1.9	2.2	1.0	2.5	0.2
Geijerene isomer	1116	—	0.2	0.2	0.2	t	t	0.4	0.3	t	t
Geijerene	1121	—	1.8	3.0	2.6	4.7	3.9	9.3	5.3	8.9	1.1
Borneol	1134	—	t	—	—	—	—	—	—	—	—
Terpinen-4-ol	1148	—	t	—	—	—	—	—	—	—	—
α-Terpineol	1159	—	0.5	—	—	—	—	—	—	—	—
Estragole	1163	2.2	—	—	—	—	—	—	—	—	—
Anisaldehyde	1200	1.9	—	—	—	—	—	—	—	—	—
cis-Anethole	1220	t	—	—	—	—	—	—	—	—	—
Isogeijerene C	1231	—	—	t	t	0.4	t	t	t	t	t
trans-Anethole	1254	92.5	2.0	t	t	t	0.9	0.8	1.1	0.6	t
Pregeijerene	1267	—	6.0	3.9	5.0	15.1	12.1	24.3	12.7	15.6	2.8
Carvacrol	1286	—	—	t	t	1.7	1.4	t	0.5	1.2	t
Dodecanol	1397	—	0.3	1.7	1.3	9.9	7.0	10.7	6.9	7.1	0.2
β-Caryophyllene	1414	—	0.2	t	t	0.3	1.2	0.3	t	t	t
trans-α-Bergamotene	1434	—	—	t	t	0.8	0.5	0.4	0.5	t	t
trans-β-Farnesene	1456	—	t	0.3	0.2	t	0.8	0.4	0.6	1.2	0.2
Dodecanol	1468	—	—	0.6	0.3	2.9	0.9	9.0	2.5	2.6	0.6
ar-Curcumene	1475	—	—	t	t	3.0	1.5	2.9	0.7	2.4	t
Zingiberene	1492	—	0.2	3.4	3.5	12.0	9.6	10.1	7.5	8.8	2.3
β-Bisabolene	1495	—	6.4	4.6	8.2	14.4	14.6	9.2	7.8	7.7	3.1
Sesquiphellandrene	1508	—	—	0.3	0.3	1.7	3.0	0.3	0.3	0.5	t
Elemol	1530	—	—	t	t	0.8	0.4	t	t	0.6	t
trans-Pseudoisoeugenyl 2-methylbutyrate	1778	0.1	4.7	3.6	2.8	t	1.9	4.0	9.0	t	4.0
cis-Epoxypseudoisoeugenyl 2-methylbutyrate	1785	—	t	t	0.1	t	t	0.4	0.2	0.7	0.2
trans-Epoxypseudoisoeugenyl 2-methylbutyrate	1814	t	70.2	52.8	56.4	2.4	16.7	5.7	21.3	0.8	78.1
Identified		96.7	93.9	75.3	82.4	77.4	80.6	92.5	79.8	65.0	93.0
Grouped Components											
Monoterpene Hydrocarbons		—	0.4								
Oxygen-Containing Monoterpenes		—	0.5	t	t	1.7	1.4	t	0.5	1.2	t
Sesquiterpene Hydrocarbons		—	6.8	8.6	12.2	32.2	31.2	23.6	17.4	20.6	5.6
Oxygen-Containing Sesquiterpenes		—		t	t	0.8	0.4	t	t	0.6	t
C_{12} Compounds		—	7.1	7.1	7.8	20.2	16.0	34.0	18.3	24.5	3.9
Phenylpropanoids		94.8	76.9	56.4	59.3	2.4	19.5	10.9	31.6	2.1	82.3
Others		1.9	1.3	3.2	3.1	20.1	12.1	24.0	12.0	16.0	1.2
Yield (v/fr. wt)		1.2	0.05	<0.05	<0.05	<0.05	<0.05	0.1	0.05	<0.05	<0.05
Yield (v/dry wt)			0.4					1.2	0.6		

* Relative to C_8–C_{19} *n*-alkanes on a DB-1 column.
t = trace (<0.05%).

Table 2. Plant species where geijerenes have been detected

Family	Plant species	Plant part	Reference
Apiaceae	*Pimpinella* sp.	Roots	[9]
Asteraceae	*Chromolaena odorata*	Leaves	[10]
	Eupatorium odoratum	Leaves	[11, 12]
Laminaceae	*Nepeta govaniana*	Aerial parts	[13]
Rosaceae	*Rubus rosifolius*	Foliage	[14]
Rutaceae	*Geijera parviflora*	Leaves	[15]
	Ruta graveolens	Roots	[16]
	Boenninghausenia albiflora	Roots	[17]
	Vepris heterophylla	Leaves	[18]

bisabolene, were not detected or only found in trace amounts, in the fruit oil. *trans*-Epoxy-pseudoisoeugenyl 2-methylbutyrate, β-bisabolene and pregeijerene were also the major components of the essential oil isolated from the roots of the parent plant, but the other reported major components of the hairy root oils were found in lower amounts in the former oil (Table 1).

The highest essential oil yield from hairy root cultures was obtained for the cultures grown in SH medium (0.05–0.1%); this yield was comparable with that obtained for the roots from the parent plant (0.05%) but it was less than the oil yield from fruits (1.2%). However, when considering hairy roots on a dry weight basis (1 g fr. wt = 80 mg dry wt), the essential oil yield of the hairy roots grown in SH medium was similar to that of the fruits but even higher than that of the roots of the parent plant (1 g fr. wt = 140 mg dry wt) (Table 1).

Studies on the accumulation of volatiles by transformed roots are scare. Nevertheless, in *Artemisia absinthium* [19] and *Valeriana officinalis* [20], the essential oil profile from the hairy roots did not match with that from the roots of the parent plant. In the present study, the essential oils isolated from anise hairy roots showed some qualitative differences, when compared with the essential oil from the parent plant roots, but the three major components of the latter oil were also major components in the oils from the transformed roots. However, quantitative differences in the percentage composition did exist and, in some cases, these were considerable, depending on the medium and growth conditions applied.

The successful transformation of *P. anisum*, together with the production of an essential oil with different profile from that of the roots of the parent plant, highlights the potential for essential oil production using this technique.

EXPERIMENTAL

Plant material

Anise plantlets were grown under aseptic conditions from commercial fruits ("seeds") (Bricobi). Fruits were soaked overnight in H_2O, prior to disinfection with 70% EtOH for 2 min, followed by a 6% NaOCl soln for 2 min. Fruits were germinated in darkness on solid Schulz medium [21] without growth regulators. After cotyledon emergence, they were transferred to a 16 h light/8 h dark photoperiod at 24°. Voucher specimens of the plant material have been deposited in the Herbarium of the Botanical Garden of Lisbon (LISU: 165574).

Hairy roots

Transformed root cultures of anise were induced by inoculation of three- to four-week-old aseptic plantlets with *A. rhizogenes* carrying the *gus* reporter gene co-integrated in the Ri plasmid and driven by a double 35S promoter (A_4 pRiA_4 70GUS). Seedlings were wounded with a scalpel and immersed for 5 min in a bacterial suspension, grown to $A_{600} = 0.9$ and diluted 1:20 (v/v) in a 0.3% sucrose soln. After careful drying on sterile filter paper, seedlings were transferred to B5/H2 medium (1:1) (B5 [22], H2 = half strength macro- and micronutrients from MS medium [23], with the same MS vitamins and sucrose content) and co-cultivated with the bacteria for 4 days. They were then transferred to fresh H2 solid medium supplemented with cefotaxime and carbenicillin, 150 μg ml^{-1} each. Medium and antibiotics were renewed every 3 weeks. Roots that developed were excised and cut in pieces for further proliferation. Transformation was confirmed by the histochemical GUS assay [24].

Determination of growth in liquid media

Four-to-8-week-old hairy roots were transferred to liquid MS/2 medium (= half strength MS) and maintained in darkness or in a 16 h light/8 h dark photoperiod at 24° on orbital shakers (80 rpm). Roots were subcultured initially every 8 days and, after complete establishment, only every two to three weeks. Four different basal media were tested to study the effect on biomass yield and essential oil composition: B5, B5/2 (= half strength B5), MS/2 and SH [25]. Before determination of growth of hairy roots, cultures were kept, at least for four subcultures, in each

of the media studied. Hairy roots growth was measured by the dissimilation method [26].

Dry weight determination

Samples of hairy roots were taken after filtration and freeze-dried for 2 days, at 10^{-1} mbar and $-42°$. Root samples of the parent plant were dried in the same way.

Isolation of essential oils

Oil samples were isolated from hairy roots with medium (preliminary studies showed no significant essential oil excretion into the medium) 8 days after subculturing, and from the fruits and roots of the parent plant, by distillation-extraction for 3 h, using a Likens–Nickerson-type apparatus with *n*-pentane as solvent, and by hydrodistillation for 3 h, using a Clevenger-type apparatus. Oil samples isolated by hydrodistillation were used to estimate oil yields and those isolated by distillation-extraction to determine oil composition, since the chance of artefact formation was considered to be less when the latter method is used.

Synthesis of pseudoeugenol and cis- *and* trans-*pseudoisoeugenol*

4-Allyloxyanisole was synthesized from 5 g 4-methoxyphenol and 3.4 ml allyl bromide in the presence of 200 ml DMSO and 2.2 g powdered KOH. The mixt. was stirred at room temp. for 1 h (yield 95%). 2-Allyl-4-methoxyphenol (pseudoeugenol) was obtained by Claisen rearrangement and purified by distillation-extraction in a Likens–Nickerson type apparatus. The pseudoisoeugenol isomers were obtained after rearrangement of pseudoeugenol under the influence of a satd soln of KOH in MeOH (1 pseudoeugenol:3 strong base soln v/v) at 160° for 1 h (yield 70% of *trans*-isomer and 30% of *cis*-isomer).

Synthesis of cis- *and* trans-*pseudoisoeugenol esters and epoxides*

The pseudoisoeugenol isomers esters were prepared from 2-methylbutyric acid chloride in pyridine and subsequently epoxidized with *m*-chloroperbenzoic acid in CH_2Cl_2 in equimolar amounts, overnight at room temp. The MS data of the synthesized products were in accordance with those of authentic compounds [27].

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. and W. Scientific Inc.) and a DB-Wax fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J. and W. Scientific Inc.). The oven temp. was programmed from 45–175° at 3° min^{-1}, subsequently at 15° min^{-1} up to 240° and then held isothermally for 10 min; injector and detector temps were 220° and 240°, respectively; carrier gas, H_2 at 30 cm s^{-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 GC peak areas without correction factors. Percentage data shown are mean values for two injections.

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. and W. Scientific Inc.) and interfaced with an ion trap detector. Oven temps were as described above; transfer line temp. 280°; ion trap temp. 220°; carrier gas He at 30 cm s^{-1}; split ratio 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40–300 mu; scan time, 1 s. The identity of components was assigned by comparison of their RIs, relative to C_8–C_{19} *n*-alkanes and MS with corresponding data of components of reference oils or of synthetic compounds.

Acknowledgements—This study was partially funded by the Anglo-Portuguese Joint Research Programme—Treaty of Windsor AI-B-8/95 and AI-B-20/96, and by the Junta Nacional de Investigação Científica e Tecnológica (JNICT) under research contract PBIC/C/BIO/1989/95. The authors gratefully acknowledge Prof. Dr M. Tepfer (INRA, France) for the *A. rhizogenes* strain (A_4 $pRiA_4$ 70GUS). Special thanks are also due to Dr Teris A. van Beck (Wageningen Agricultural University, The Netherlands) for an authentic sample of geijerenes.

REFERENCES

1. Simon, J. E., Chadwick, A. F. and Craker, L. E., In *Herbs, an Indexed Bibliography*, 1971–1980. Elsevier, Amsterdam, 1984, pp. 5–6.
2. Ernst, D., in *Biotechnology in Agriculture and Forestry*, Vol. 7, *Medicinal and Aromatic Plants II*, ed. Y. P. S. Bajaj, Springer-Verlag, Berlin, Heidelberg, 1989, pp. 381–397.
3. Kubeczka, K.-H., von Massow, F., Formacek, V. and Smith, M. A. R., *Z. Naturforsch.*, 1976, **31b**, 283.
4. Merkel, B. and Reichling, J., *Z. Naturforsch.*, 1990, **45c**, 602.
5. Salem, K. M. S. A. and Charlwood, B. V., *Plant Cell Tiss. Org. Cult.*, 1995, **40**, 209.
6. Carter, G. T., Schnoes, H. K. and Lichtenstein, E. P., *Phytochemistry*, 1977, **16**, 615.
7. Bottini, A. T., Dev, V., Garfagnoli, D. J.,

Mathela, C. S., Melkani, A. B., Miller, A. A. and Sturm, N. S., *Phytochemistry*, 1986, **25**, 207.
8. Dev, V. and Bottini, A. T., *J. Nat. Prod.*, 1987, **50**, 968.
9. Kubeczka, K.-H., Bohn, I. and Schultze, W., *Z. Naturforsch.*, 1989, **44c**, 177.
10. Duñg, N. X., Bien, L. K. and Leclercq, P. A., *J. Essent. Oil. Res.*, 1992, **4**, 309.
11. Lamaty, G., Menut, C., Amvam Zollo, P.-H., Kuiate, J. R., Bessière, J.-M., Ouamba, J. M. and Silou, T., *J. Essent. Oil Res.*, 1992, **4**, 101.
12. Bamba, D., Bessière, J.-M., Marion, C., Pélissier, Y. and Fourasté, I., *Planta Med.*, 1993, **59**, 184.
13. Mathela, C. S., Kharkwal, H. and Laurent, R., *J. Essent. Oil Res.*, 1994, **6**, 425.
14. Southwell, I. A., *Aust. J. Chem.*, 1978, **31**, 2527.
15. Jones, R. V. H. and Sutherland, M. D., *Aust. J. Chem.*, 1968, **21**, 2255.
16. Tattje, D. H. E. and Bos, R., *Pharm. Weekbl.*, 1972, **107**, 261.
17. Parihar, R., Shah, G. C. and Mathela, C. S., *Fitoterapia*, 1991, **62**, 277.
18. Moulis, C., Fouraste, I., Keita, A. and Bessière, J.-M., *Flavour Fragr. J.*, 1994, **9**, 35.
19. Kennedy, A. I., Deans, S. G., Svoboda, K. P., Gray, A. I. and Waterman, P. G., *Phytochemistry*, 1993, **32**, 1449.
20. Gränicher, F., Christen, P. and Kapetanidis, I., *Phytochemistry*, 1995, **40**, 1421.
21. Schulz, A., *Ph.D. Thesis*, Technische Universität, Hannover.
22. Gamborg, O. L., Miller, R. A. and Ojima, K., *Exp. Cell Res.*, 1968, **50**, 151.
23. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473.
24. Jefferson, R. A., *Plant Mol. Biol. Rep.*, 1987, **5**, 387.
25. Schenk, U. R. and Hildebrandt, A. C., *Can. J. Bot.*, 1972, **50**, 199.
26. Schripsema, J., Meijer, A. H., van Iren, F., ten Hoopen, H. J. C. and Verpoorte, R., *Plant Cell Tiss. Org. Cult.*, 1990, **22**, 55.
27. Martin, R., Reichling, J. and Becker, H., *Planta Med.*, 1985, **51**, 198.