BIOTECHNOLOGY LETTERS
Volume 18 No. 8 (August) pp.863-868
Received as revised 28 May

BIOTRANSFORMATION OF MONOTERPENES AND SESQUITERPENES BY CELL SUSPENSION CULTURES OF *ACHILLEA MILLEFOLIUM* L. SSP. *MILLEFOLIUM*

A. Cristina Figueiredo[1]*, M. João Almendra[1], José G. Barroso[1], Johannes J. C. Scheffer[2]

[1] Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1780 Lisbon, Portugal

[2] Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands

Abstract

The transformation capacity of *Achillea millefolium* L. ssp. *millefolium* (yarrow) cell suspension cultures was investigated using geraniol (50mg/l) and borneol, menthol, thymol and farnesols (25mg/l) as substrates. Apart from converting these substrates into several biotransformation products, the cell suspension cultures were also able to glycosylate both the substrates and the biotransformation products.

Key Words *Achillea millefolium* L. ssp. *millefolium* Yarrow Compositae Biotransformation Glycosylation Geraniol Borneol Menthol Thymol Farnesols

Introduction

Biotransformation using plant cell cultures has received increasing attention as a method for the conversion of monoterpenes, diterpenes, steroids, triterpenes, alkaloids and some synthetic chemicals, to produce more useful substances (Barz and Ellis 1981; Suga and Hirata 1990; Hamada 1993; Hirata *et al.* 1994; Hamada *et al.* 1994; Orihara and Furuya 1994a,b; Orihara *et al.* 1994). Glycosylation is a common biotransformation reaction that has some industrial application, mainly in the pharmaceutical and food industries (Berger and Drawert 1988).

In vivo glycosylation is a part of the normal metabolic turnover. Since plant cell cultures do not have specialized storage structures, *in vitro* glycosylation can be considered as a detoxification mechanism. Compounds such as limonene, α-phellandrene, α- and β-pinene, 1,8-cineole, camphor, geraniol, nerol, linalol, citral, carvone and isomenthone are toxic to the cells, and although the tolerance of the cultured cells to these compounds varies during the cell cycle, a concentration of 0.5 mg/ml (=1.5 mg/g fresh weight) in the initial growth stages is enough to induce cell death (Charlwood *et al.* 1988; Falk *et al.* 1990; Orihara and Furuya 1994b; Orihara *et al.* 1994). Moreover, according to Berger and Drawert (1988) cultured cells can transform foreign substrates to carbon and energy, followed by a transfer to primary pathways. The glycosides would in this way facilitate the transport to the catabolic enzymatic sites.

As a part of our studies on *Achillea millefolium* L. ssp. *millefolium* (Compositae) cell suspension cultures we

investigated their capacity to biotransform monoterpenes and sesquiterpenes.

Materials and Methods

Cell suspension cultures: A. millefolium L. ssp. *millefolium* cell suspension cultures were maintained in darkness as previously reported (Figueiredo and Pais 1991).

Biotransformation experiments: Substrate-methanol mixture (2% v/v) was added to each culture flask, 5 days following subculture, to give the substrate at 50mg/l culture medium for geraniol and 25mg/l culture medium for borneol, menthol, thymol, and farnesol (mixture of isomers). Substrate evaporation control experiments were performed by adding the same amount of substrate to flasks containing only basal culture medium, and keeping them in the same conditions as the culture flasks throughout the experiment culture period.

Growth measurements: The influence of the different substrates on the cell growth was determined by means of the dissimilation method (Schripsema *et al.* 1990).

Isolation Procedure: The cell suspension cultures were harvested periodically during the growth cycle; an internal standard (α-terpineol-CH_2Cl_2, 8% v/v) was added prior to extraction, in a concentration of 16mg/l for the experiments with geraniol and 8mg/l for the other substrates. The volatile biotransformation products were isolated by distillation-extraction, for 3h, using a Likens-Nickerson-type apparatus with *n*-pentane as organic solvent.

Determination of glycosidic bound volatiles: β-Glycosidase (4U/mg, 1g/l) was added to the remaining distillation-extracted aqueous solution according to the method of Baerheim Svendsen and Merkx (1989). The hydrolysis was performed for 20h at 37°C, and the isolation of the volatiles obtained after hydrolysis was done as above.

Gas Chromatography: GC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with two FIDs, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30m x 0.25mm i.d., film thickness 0.25μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-Wax fused-silica column (30m x 0.25mm i.d., film thickness 0.25μm; J & W Scientific Inc.). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 240°C, and then held isothermal for 10min; injector and detector temperatures, 220°C and 240°C, respectively; carrier gas, H_2, adjusted to a linear velocity of 30cm/s. The samples were injected using the split sampling technique, ratio 1:50. The concentration of both the substrates and of the biotransformation products was determined by the internal standard method.

Gas Chromatography-Mass Spectrometry: The GC-MS unit consisted of a Carlo Erba 6000 Vega gas chromatograph, equipped with a DB-1 fused-silica column (30m x 0.25mm i.d., film thickness 0.25μm; J & W Scientific Inc.), and interfaced with a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). Oven temperature was as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30cm/s; splitting ratio, 1:40; ionization energy, 70eV; ionization current, 60μA; scan range, 40-300u; scan time, 1s. The identity of the biotransformation products was assigned by comparison of their retention indices, relative to *n*-alkanes, and mass spectra with corresponding data of components of reference essential oils.

Results and Discussion

Preliminary experiments with yarrow cell suspension cultures showed that the moment of addition of the substrates (either at the time of subculturing or five days later) did not affect significantly the cell growth, Fig. 1, nor the type of biotransformation products. Only the biotransformation capacity was slightly retarded when the substrate was added at the time of subculturing, probably because it is a time of intense culture growth. The prior studies also showed that the cultures could withstand higher amounts of substrates (100mg/l and 50mg/l), still without critically influencing the cell growth. Nevertheless, as was observed in these experiments, when using borneol, menthol or thymol at 25mg/l, the substatres were never completely biotransformed by the cultures. Independently of the added substrate, the constitutive volatiles of the *A. millefolium* cell suspension cultures (Figueiredo *et al.* 1995) were always detected in varying amounts, together with the substrates and biotransformation products.

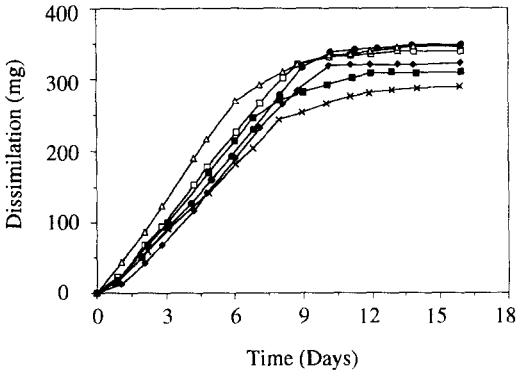

Fig. 1: Dissimilation growth curves of the cell suspension cultures after addition of geraniol (Δ), borneol (◆), menthol (□), thymol (×), farnesols (■) five days after subculturing, and of control cultures (= without substates; ●).

The evaporation control experiments showed that there was no marked evaporation of the substrates during the culture period, nor did they decompose into any of the biotransformation products.

Nerol, neral and geranial were the main compounds detected after the incubation with geraniol (Table 1), Fig. 2. Myrcene, limonene, *cis*-β-ocimene, *trans*-β-ocimene, γ-terpinene, *cis*-linalol oxide, *trans*-linalol oxide, *cis*-rose oxide, *trans*-rose oxide and nerol oxide were also detected, but always in amounts lower than 0.05μg/ml, Fig. 3. The common interconversion of geraniol into nerol, or the oxidation of these alcohols into their respective aldehydes, detected after incubation with geraniol, has also been recorded for cell suspension cultures of *Euphorbia characias*, *Nicotiana tabacum*, *Catharanthus roseus*, *Glycine max* (Carriere *et al.* 1989) and *Petroselinum crispum* (Gbolade and Lockwood 1989).

Table 1. Variation in the amount of substrate and main biotransformation products after the addition of geraniol to *Achillea millefolium* cell suspension cultures. t = trace (<0.05μg/ml).

Component	Time after addition of geraniol (h)				
(μg/ml)	1	8	23	35	49
Nerol	6.4	4.4	1.5	t	t
Neral	2.9	1.4	0.4	t	t
Geraniol	46.0	13.1	2.4	0.8	t
Geranial	3.7	1.1	0.3	t	t

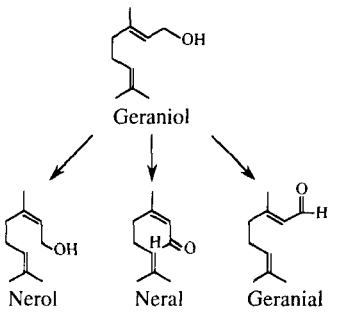

Fig. 2: Main biotransformation products detected after incubation with geraniol.

Two days after the addition (= at day 7), geraniol as well as its main transformation products were no longer detected during the cell culture growth, until when the cells came into the late stationary phase, a period of cell lysis.

In measuring substrate disappearance in cell-free medium, we found that the evaporation was too low to account for the disappearance of the substrate and of the products; therefore, experiments were performed to determine whether the substrate and the transformation products were accumulated in a glycosylated form. In fact, the main biotransformation products along with the substrate, and α-pinene and other hydrocarbons, were detected following enzymatic hydrolysis already one hour after the substrate addition. However, the biotransformation products and α-pinene were only detected in concentrations higher than trace amounts (nerol and neral 0.4μg/ml, geranial 0.3μg/ml, α-pinene 0.1μg/ml and geraniol traces) 48 hours after substrate addition.

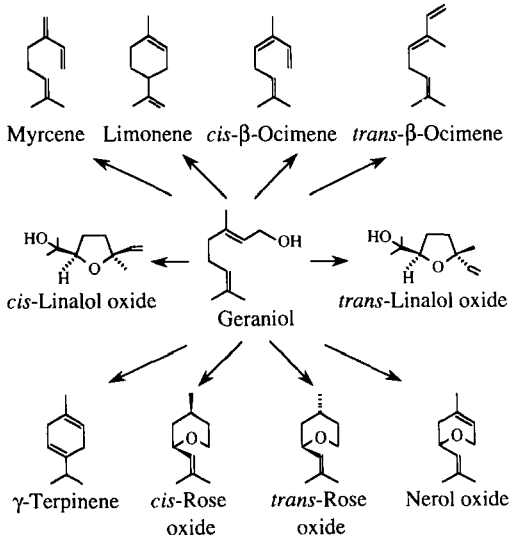

Fig. 3: Minor biotransformation products detected after incubation with geraniol.

Following borneol administration to the yarrow cultures, camphor and isoborneol were the biotransformation products detected (Table 2), Fig. 4. Similarly to geraniol, a part of the added substrate as well as of the transformation products was found to be glycosylated; on hydrolysis, the compounds were always present in trace amounts. The capability of biotransforming this bicyclic monoterpene seems to be species specific, since plant cell cultures of *Nicotiana tabacum* were able to biotransform it (Suga *et al.* 1983), whereas those of *Cannabis sativa* did not (Itokawa *et al.* 1977) and those of *Eucalyptus perriniana* were seen to glycosylate it into six new biotransformation products (Orihara and Furuya 1992).

Table 2. Variation in the amount of substrate and main biotransformation products after the addition of borneol to *Achillea millefolium* cell suspension cultures. t = trace (<0.05μg/ml).

Component	Time after addition of borneol (h)					
(μg/ml)	1	6	24	46	98	216
Camphor	t	0.1	0.4	0.7	0.1	t
Isoborneol	0.3	0.2	0.1	0.1	0.2	t
Borneol	20.8	16.4	20.1	28.5	2.2	1.1

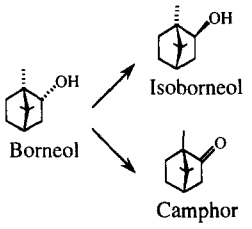

Fig. 4: Biotransformation products detected after incubation with borneol.

The biotransformation products detected after the incubation with menthol, namely menthone and menthyl acetate, were always found in trace amounts (Table 3), Fig. 5. After enzymatic hydrolysis, α-pinene (up to 8.1μg/ml), β-pinene (up to 0.6μg/ml),

1,8-cineole (up to 0.5µg/ml), limonene (up to 1.0µg/ml), pinocarvone (up to 8.3µg/ml) and menthol (up to 1.7µg/ml) were found, but in the late stationary phase, i. e. 12 days after subculturing. Menthyl glycosides were also important transformation products from *Eucalyptus perriniana* cell cultures, probably due to a cellular defence mechanism (Furuya *et al.* 1989; Orihara *et al.* 1991). In *Mentha piperita* cell cultures, the formation of menthyl glycosides was dependent on the concentration of substrate, incubation period, cell age and aggregation, and light (Berger and Drawert 1988).

Table 3. Variation in the amount of substrate after the addition of menthol to *Achillea millefolium* cell suspension cultures. t = trace (<0.05µg/ml).

Component	Time after addition of menthol (h)					
(µg/ml)	1	6	24	46	98	216
Menthol	14.8	9.2	3.2	0.8	0.6	0.2

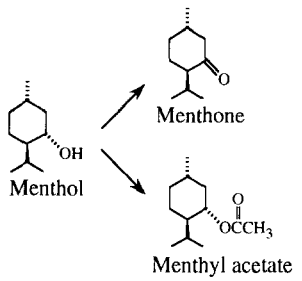

Fig. 5: Biotransformation products detected after incubation with menthol.

Trace amounts of 1,8-cineole, limonene and carvacrol were detected throughout the culture period, after the addition of thymol to the cell suspension culture (Table 4), Fig. 6. The same biotransformation products, occurring again in trace amounts, along with α-pinene, terpinolene and thymol were isolated after the enzymatic hydrolysis procedure (Table 5).

Table 4. Variation in the amount of substrate after the addition of thymol to *Achillea millefolium* cell suspension cultures. t = trace (<0.05µg/ml).

Component	Time after addition of thymol (h)				
(µg/ml)	1	20	44	78	292
Thymol	17.8	1.0	2.7	1.8	0.3

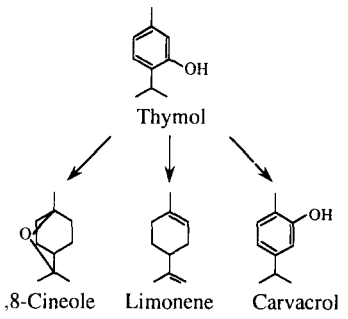

Fig. 6: Biotransformation products detected after incubation with thymol.

Table 5. Variation in the amount of substrate and main biotransformation products after enzymatic hydrolysis of the remaining distillation-extracted solution from the thymol added cultures. t = trace (<0.05µg/ml).

Component	Time after addition of thymol (h)				
(µg/ml)	1	20	44	78	292
α-Pinene	0.8	0.1	0.3	t	t
Terpinolene	0.1	t	t	0.1	t
Thymol	0.3	3.5	1.5	0.6	t

Table 6. Variation in the amount of substrate after the addition of farnesols to *Achillea millefolium* cell suspension cultures. t = trace (<0.05µg/ml).

Component (µg/ml)	Time after addition of farnesols (h)			
	1	23	49	83
cis, trans-Farnesol	0.9	0.5	t	t
cis-Farnesol+*trans*-Farnesol	2.5	0.5	t	t
trans, cis-Farnesol	0.9	t	t	t

Two days after the addition of the farnesols, the mixture of isomers was only detected in trace amounts (Table 6), Fig. 7. Likewise, the biotransformation products, *cis*-β-farnesene, *trans*-β-farnesene and α-*trans, trans*-farnesene, were found in trace

amounts, either in the free form or after enzymatic hydrolysis, from one hour after addition of the substrate and then throughout the culture period.

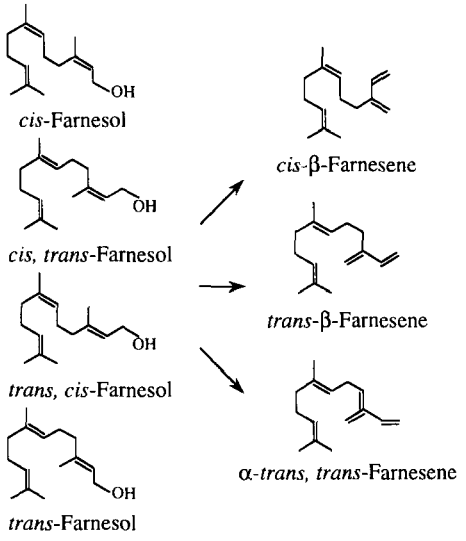

Fig. 7: Biotransformation products detected after incubation with the mixture of farnesol isomers

In conclusion, yarrow cell suspension cultures revealed the capacity to biotransform both monoterpenes and sesquiterpenes, although with different degrees of efficiency. Part of the added substrates and biotransformation products was converted into, and accumulated as glycosylated compounds. This glycosylation mechanism can be regarded as a detoxification process and as a way of converting compounds into more readily usable forms, which may explain the disappearance of the glycosylated compounds as the cultures aged.

Acknowledgements - This study was partially granted by the Centro de Biotecnologia Vegetal of the Instituto de Biotecnologia e Química Fina (IBQF) Lisbon.

References

Baerheim Svendsen A, Merkx IJM (1989) Planta Med. 55: 38-40

Barz W, Ellis BE (1981) In: Natural products as medicinal agents, Suppl. Planta Med. and Lloydia. Hippokrates Verlag, Berlin, pp 471-607

Berger RG, Drawert F (1988) Z. Naturforsch. 43c: 485-490

Carriere F, Gil G, Tapie P, Chagvardieff P (1989) Phytochemistry 28: 1087-1090

Charlwood BV, Brown JT, Moustou C, Morris GS, Charlwood KA (1988) In: Schreier P (ed) Bioflavour'87, Walter de Gruyter, Berlin, pp. 303-314

Falk KL, Gershenzon J, Croteau R (1990) Plant Physiol. 93: 1559-1567

Figueiredo AC, Pais MSS (1991) Biotechnology Letters 13: 63-68

Figueiredo AC, Pais MSS, Scheffer JJC (1995) Plant Cell Tiss. Org. Cult. 40: 113-118

Furuya T, Orihara Y, Miyatake H (1989) J. Chem. Soc. Perkin Trans. I: 1711-1719

Gbolade AA, Lockwood GB (1989) Z. Naturforsch. 44c: 1066-1068

Hamada H (1993) Proceedings of 1st Asia-Pacific Conference on Plant Cell and Tissue Culture.

Hamada H, Fuchikami Y, Ikematsu Y, Hirata T, Williams HJ, Scott AI (1994) Phytochemistry 37: 1037-1038

Hirata T, Ikeda Y, Izumi S, Shimoda K, Hamada H, Kawamura T (1994) Phytochemistry 37: 401-403

Itokawa H, Takeya K, Mihashi S (1977) Chem. Pharm. Bull. 25: 1941

Orihara Y, Miyatake H, Furuya T (1991) Phytochemistry 30: 1843-1845

Orihara Y, Furuya T (1992) Phytochemistry 34: 1045-1048

Orihara Y, Furuya T (1994a) Phytochemistry 36: 55-59

Orihara Y, Furuya T (1994b) Phytochemistry 35: 641-644

Orihara Y, Noguchi T, Furuya T (1994) Phytochemistry 35: 941-945

Schripsema J, Meijer AH, van Iren F, ten Hoopen HJC, Verpoorte R (1990) Plant Cell Tiss. Org. Cult. 22: 55-64

Suga T, Hirata T., Hamada H, Futatsugi M (1983) Plant Cell Reports 2: 186-188

Suga T, Hirata T (1990) Phytochemistry 29: 2393-2406.