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# BIOTRANSFORMATION OF MONOTERPENOIDS BY SUSPENSION CULTURES OF *LAVANDULA ANGUSTIFOLIA*

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**Key Word Index**—*Lavandula angustifolia*; Labiateae; lavender; plant tissue culture; biotransformation; monoterpenoids; reductase.

**Abstract**—Suspension cultures of *Lavandula angustifolia* reduced monoterpenoid aldehydes and structurally related compounds to their corresponding primary alcohols. Acyclic primary monoterpenoid alcohols were further metabolized to unidentified compounds. Cyclic, secondary, tertiary and aromatic alcohols were not metabolized.

## INTRODUCTION

The biotransformation of monoterpenoids by plant tissue culture has attracted recent attention. Cultures of *Vitis vinifera* [1], *Nicotiana tabacum* [2–8], *Cannabis sativa* [9, 10], *Catharanthus roseus* [11] and *Mentha* species [12–14] have been examined. Here, we report on the ability of suspension cultures of *L. angustifolia* to reduce monoterpenoid aldehydes and structurally related compounds. Acyclic monoterpenoid primary alcohols, once formed, disappeared from the cultures in a manner similar to that previously reported for the disappearance of neomenthol from *Mentha* cultures [12].

## RESULTS AND DISCUSSION

The callus cultures of *L. angustifolia* grew rapidly, nearly trebling their fresh weight in 2 weeks. A blue pigment accumulated in the agar medium. This pigment has recently been identified as the (*Z,E*)-2-(3,4-dihydroxyphenyl)ethenyl ester of 3-(3,4-dihydroxyphenyl)-2-propenoic acid and its (*E,E*)-isomer. The excreted product complexes with $Fe^{2+}$ in the medium, producing the blue coloration [15]. The suspension culture grew as a fine suspension, reaching a maximum cell density of about $3 \times 10^6$ cells/cm$^3$ in 5 days ($t_d$ = 20 hr, $\mu = 0.83\ day^{-1}$). No blue pigment was seen in the suspension cultures and they did not produce any monoterpenoids or their glycosides.

A selection of monoterpenoids was added individually to the suspension cultures and after incubation the products were extracted with diethyl ether and analysed by GC/MS. The monoterpenoid aldehydes citral (geranial and neral in the proportions of 65 and 35%, respectively), citronellal and perillaldehyde were reduced to their corresponding alcohols and the time courses for the reductions are shown in Figs. 1a–1c. The aromatic aldehydes benzaldehyde and cinnamaldehyde were also reduced but more slowly (Fig. 1d). Octanal and monoterpenoid ketones were not reduced. The rates at which acyclic, cyclic and aromatic aldehydes were reduced by the cultures of *L. angustifolia* (Figs. 1a–1d) were widely different, suggesting the presence of a semi-specific reductase. The specificity of monoterpenoid catabolic enzymes has been the subject of recent debate. Optically selective menthone dehydrogenases from *Mentha piperita* have been shown to accept structurally related substrates [16]. Monoterpenoid alcohol dehydrogenases from *Foeniculum vulgare* and *Tanacetum vulgare* have also been shown to be semi-specific [17].

Separate examination of the cells and the medium revealed that all the product alcohols were present in the celluar fraction.

Geraniol, nerol and citronellol, once formed by the reduction of the corresponding aldehydes, were found to disappear from the cultures over about 15 hr. The time courses for the disappearance of geraniol and citronellol can be seen in Fig. 2. In contrast, the aromatic alcohols benzyl alcohol and cinnamyl alcohol, the cyclic monoterpenoid primary alcohol perillyl alcohol, the secondary alcohol menthol and the acyclic monoterpenoid tertiary alcohol linalool remained unmetabolized in the cultures over 72 hr. Neomenthol is glucosylated in *Mentha piperita* rhizomes [18] and monoterpenoid glucosides are commonly found in the essential oil-producing plants (e.g. refs. [19, 20]). The possibility that the disappearance of the monoterpenoid alcohols was due to their glucosylation was therefore investigated by attempted recovery of the parent alcohol from the cultures after acid hydrolysis. The acid hydrolysis was carried out after 3, 8 and 15 hr incubation which corresponded to the disappearance of 60, 85 and 100% of the substrate, respectively. Unmetabolized alcohol was extracted with diethyl ether prior to acid hydrolysis of the aqueous phase. Parent alcohols were not recovered from the cultures after acid hydrolysis and when aqueous extracts, prepared from unhydrolysed cultures, were examined by TLC they did not reveal the presence of any substances with $R_f$ values appropriate to monoterpenoid glucosides. There was no evidence therefore that a glucosylated adduct was accumulated in the cultures, although it was possible that

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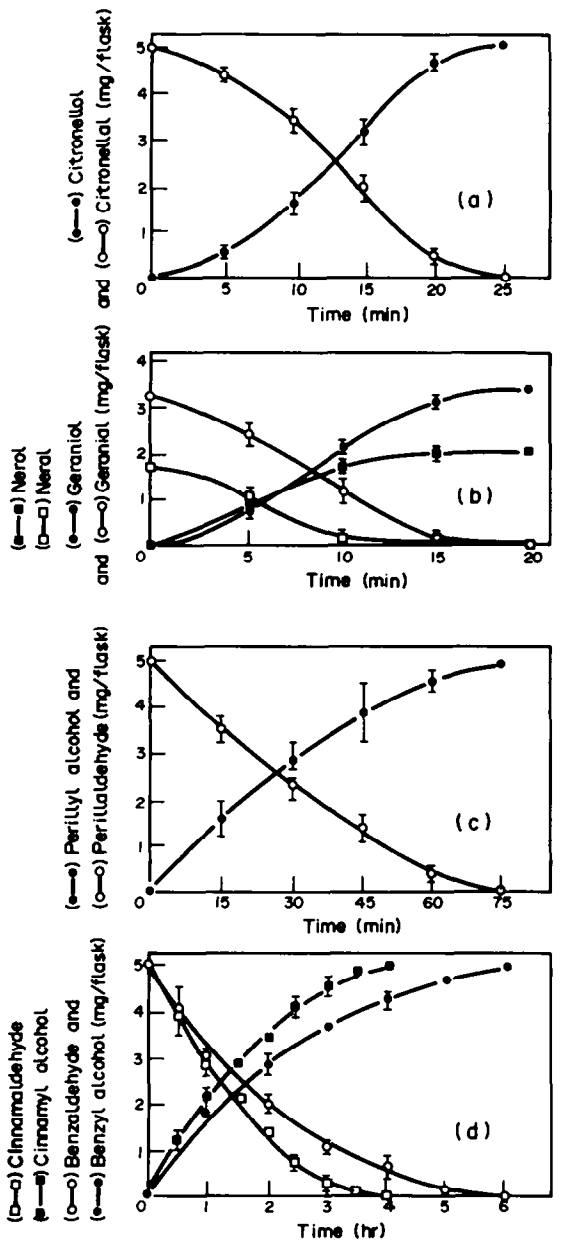


Fig. 1. Time courses for the reductions of citronellal (a), neral and geranial (b), perrilaldehyde (c), benzaldehyde and cinnamaldehyde (d) to their corresponding alcohols by suspension cultures of *L. angustifolia* (100 $cm^3$/flask). Standard deviations for the reductions of citral and citronellal were calculated from six separate results and the standard deviations for the reductions of perillaldehyde, benzaldehyde and cinnamaldehyde were calculated from four separate results.

such an adduct, having been formed, was itself rapidly metabolized. Glucosylation is an important step in the catabolism of camphor in *Salvia officinalis* [21] and neomenthol in *Mentha piperita* [22].

Monoterpenoids are subject to metabolic flux [23]. Possible enzymatic processes to account for the catabolism of monoterpenoids have only recently been suggested [24].

The addition of (±)-linalyl and (±)-bornyl acetates to

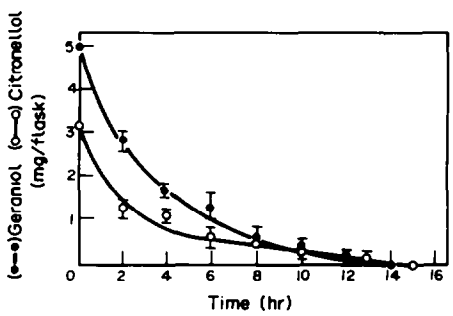


Fig. 2. Time course for the disappearance of geraniol and citronellol from cultures of *L. angustifolia* (100 $cm^3$/flask). Standard deviations were calculated from four separate results.

the cultures resulted in their hydrolysis to form mixtures of the acetates and parent alcohols in approximately equal amounts. The ratio of acetate to alcohol was unchanged after 72 hr incubation. The apparent steady state may have been due to the optically selective hydrolysis of the acetate moiety. Optically selective menthyl acetate hydrolases have been reported to exist in micro-organisms [25].

With only a few exceptions [26], it is widely recognized that monoterpenoids are not produced in undifferentiated cultures. The absence of monoterpenoids in unorganized cultures has been attributed to the lack of specialized oil glands, which may be the sites of synthesis in the intact plant [27]. The enzymes responsible for monoterpenoid anabolism are present in some non-producing cultures [23]. The results presented here and elsewhere [12] raise the possibility that the absence of monoterpenoids in undifferentiated cultures may be due to their catabolism immediately following their formation. In the intact plant the accumulation of essential oil in metabolically remote oil glands and the seasonal appearance of catabolic enzymes [24] may control the rate of turnover and thereby the composition of the oil. Such controls may not exist in cultures.

## EXPERIMENTAL

*GC/MS* was performed using a Hewlett–Packard 5992 benchtop instrument fitted with a glass column (3 mm × 1.5 m) packed with 20% Carbowax 20M on Chromosorb w (80–100 mesh) and programmed at 60–100° (5°/min); carrier gas was He at 30 $cm^3$/min. The GC was coupled to a quadrapole mass spectrometer which recorded spectra over a *m/z* range of 20–350 at a scan speed of 380 mu/sec with a delay of 0.5 sec between scans. The electron multiplier was 3 kV.

*Cultures of* Lavandula angustifolia *Mill.* were established on Murashige and Skoog medium (M/S) [28] supplemented with 3% (w/v) sucrose, 2% (w/v) agar, 9 mM 2,4-dichlorophenoxyacetic acid and 0.47 mM kinetin. The callus was subcultured onto fresh medium fortnightly. About 2 g of callus was subcultured into liquid medium (callus medium without agar) and incubated (25°) in a reciprocal shaker (120 rpm) in the dark. The culture was subcultured (25% v/v inoculum) weekly and had been maintained for over 1 year.

*Substrates.* Benzaldehyde (98%), benzyl alcohol (98%), (±)-camphor (97%), (*E*)-cinnamaldehyde (99%), cinnamyl alcohol (98%), citral (95% overall, 65% geranial + 35% neral), (±)-linalool (99%), (±)-menthol (99%), (−)-menthone (90%), myrcene (technical grade), (*S*)-(−)-perillaldehyde (96%), (*S*)-(−)-

perillyl alcohol (85%), (±)-α-pinene (98%), (S)-(−)-β-pinene (98%) and octanal (99%) were obtained from the Aldrich Chem. Co. (±)-Bornyl acetate (95%), (±)-citronellal (95%), (±)-citronellol (90%) and (±)-linalyl acetate were generous gifts from Treat and Co. Ltd., Bury St. Edmunds, Suffolk, U.K.

*Incubation of substrates with the suspension cultures* was carried out using 100 $cm^3$ of culture (5 days old) to which 5 mg of substrate was added without prior sterilization. Controls were prepared by the addition of 5 mg of substrate to 100 $cm^3$ of M/S medium and in the case of geraniol, citral, citronellal and citronellol, 5 mg was added to 100 $cm^3$ of heat-killed culture (70° for 10 min). Time courses were obtained by taking duplicate flasks for analysis at each of the time points shown in Figs. 1 and 2. Linalool (5 mg) was added to each flask, prior to extraction, as an internal standard. The amount of unmetabolized substrate and the amount of alcohol product were calculated by comparing the area under their chromatographic peaks with that of the respective peak for the internal standard (linalool).

*Isolation and identification of the products* were achieved by extracting the cultures with equal vols. of $Et_2O$ for 2 hr whilst being agitated on an orbital shaker (120 rpm). The $Et_2O$ extracts were reduced to 3 $cm^3$ by a stream of $N_2$ at 40°, dried ($MgSO_4$) and analysed by GC/MS coupled to a database and computerized library search facility. The ten most abundant ions of each spectrum were compared with a Hewlett–Packard 5882A flavour-fragrance library (correlation = 0.85). Identification was confirmed by matching the GC retention times and the MS with authentic samples. The time courses for the reduction of citral and citronellal were rapid and the addition of $Et_2O$ alone was insufficient to stop the reaction immediately. The cultures were therefore frozen in liquid $N_2$ at the appropriate time point (Figs. 1a and 1b), an equal vol. of $Et_2O$ was added and the cultures were allowed to thaw (25°) over 4 hr whilst being agitated as described above.

*Acid hydrolysis.* Geraniol (20 mg) was added to the suspension cultures (500 $cm^3$/flask) and incubated for 3, 8 and 15 hr. At each time point a flask was removed, 20 mg linalool was added (internal standard) and the culture extracted (3 ×) with 0.5 vol. $Et_2O$. The final extract was retained for analysis. The culture was then made 0.5 M with HCl, refluxed for 1 hr and re-extracted with 0.5 vol. $Et_2O$. The $Et_2O$ extracts obtained before and after acid hydrolysis were analysed by GC/MS and the amounts of geraniol in each were quantified against the linallol standard. Recovery of the parent alcohol would result in an increase in the amount of geraniol present in the extract obtained after acid hydrolysis.

*Geraniol-β-D-glucoside* was prepared according to ref. [29]. A mixture of glucose (5 g), geraniol (4 g), $Me_2CO$ (5 $cm^3$), 0.1 M acetate buffer (pH 5.5) and β-glucosidase (*ex* almond, Sigma Chem. Co.) (200 units) was incubated at 15° for 12 weeks.

*TLC.* Geraniol (30 mg) was incubated with suspension cultures (500 $cm^3$/flask) for 3, 8 and 15 hr. At each time point, excess geraniol was removed by extraction with 3 × 0.5 vol. $Et_2O$. The $Et_2O$ extracts were pooled and washed (2 ×) with an equal vol. of $H_2O$. The washings were combined with the aq. phase and reduced *in vacuo* (*ca* 80°) to *ca* 10 $cm^3$, which was then filtered through a Whatman No. 1 filter paper. This filtrate, geraniol-β-D-glucoside standard, geraniol (2% in $Et_2O$) and glucose (1% aqueous) (*ca* 60, 30, 1.5 and 3 μl, respectively) were spotted onto silica gel-H commercial TLC plates and developed in PrOH–EtOAc–$H_2O$ (6:3:1). Compounds were visualized with 1% vanillin in 50% EtOH–$H_2SO_4$ (100° for 5 min).

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## REFERENCES

1. Amid, C., Moisseeff, M. and Fallot, J. (1982) *Plant Cell Rep.* **1**, 91.
2. Suga, T., Hirata, T., Hirano, Y. and Ito, T. (1976) *Chem. Letters* 1245.
3. Hirata, T., Aoki, T., Hirano, Y., Ito, T. and Suga, T. (1981) *Chem. Letters* 3527.
4. Suga, T., Aoki, T., Hirata, T., Lee, Y. and Nishimura, O. (1980) *Chem. Letters* 229.
5. Hirata, T., Lee, Y. and Takayuki, S. (1982) *Chem. Letters* 671.
6. Lee, Y., Hirata, T. and Suga, T. (1983) *J. Chem. Soc. Perkin Trans.* **I**, 2475.
7. Hirata, T., Hamada, H., Aoki, T. and Suga, T. (1982) *Phytochemistry* **21**, 2209.
8. Suga, T., Hirata, T. and Futatsugi, M. (1984) *Phytochemistry* **23**, 1327.
9. Itokawa, H., Takeya, K. and Mihashi, S. (1977) *Chem. Pharm. Bull.* **25**, 1941.
10. Itokawa, H., Takeya, K. and Akasu, M. (1976) *Chem. Pharm. Bull.* **24**, 1681.
11. Balsevich, J. (1984) *Planta Med.* **2**, 128.
12. Aviv, D., Krochmal, E. and Galun, E. (1981) *Planta Med.* **42**, 236.
13. Aviv, D. and Galun, E. (1978) *Planta Med.* **33**, 70.
14. Aviv, D., Krochmal, E. and Galun, E. (1983) *Planta Med.* **47**, 7.
15. Banthorpe, D. V., Bilyard, H. J. and Watson, D. G. (1985) *Phytochemistry* **24**, 2677.
16. Kjonaas, R., Taylor, C. and Croteau, R. (1982) *Phytochemistry* **19**, 1343.
17. Croteau, R. and Felton, M. (1980) *Phytochemistry* **19**, 1343.
18. Croteau, R. and Martinkus, C. (1979) *Plant Physiol.* **64**, 169.
19. 
20. Francis, M. J. O. and Allcock, C. (1969) *Phytochemistry* **8**, 1339.
21. Croteau, R., El-Baily, H. and El-Hindawali, S. (1984) *Arch. Biochem. Biophys.* **228**, 667.
22. Croteau, R. and Sood, V. K. (1985) *Plant Physiol.* **77**, 801.
23. Burbott, J. and Loomis, D. (1969) *Plant Physiol.* **44**, 173.
24. Banthorpe, D. V. and Osborne, M. J. (1984) *Phytochemistry* **23**, 905.
25. Schindler, J. and Schmid, R. D. (1982) *Process Biochem.* **17**, 2.
26. Watts, M. J., Galpin, I. J. and Collin, H. A. *New Phytol.* **100**, 45.
27. Croteau, R. (1977) *Plant Physiol.* **59**, 519.
28. Murashige, T. and Skoog, F. (1982) *Physiol. Plant.* **15**, 473.
29. Bourquelot, E. and Bridel, M. (1913) *C.R. Acad. Sci. Paris* **157**, 72.