# CHAPTER IV
# RESULTS

## 4.1 Identification of essential oil obtained by hydrodistillation

The essential oil constituents of individual plant species obtained by hydrodistillation have been listed below in Table 16-20.

### 4.1.1 *Artemisia vulgaris* var. *indica*

The essential oil constituents of *Artemisia vulgaris* var. *indica* had been obtained by fresh leaves hydrodistillation. The yield had been found to be 0.25% (v/w) of fresh weight and the constituents identified by GC-MS had been shown as six monoterpenes, seven oxygenated monoterpenes, four sesquiterpenes, and six oxygenated sesquiterpenes (Table 16). Amongst these compounds, davanone appeared to be the major constituents (71.56%), followed by chrysanthenone (6.65%) and 9-epi-β-caryophyllene (3.80%). The list of essential oil compositions has been shown in Table 16.

### 4.1.2 *Cuminum cyminum*

The essential oil of *Cuminum cyminum* had been obtained by fruits hydrodistillation. The oil yield was found to be 0.40 % (v/w) of fresh weight. Thirty four compounds was analysed by GC-MS and identified as eleven monoterpenes, fifteen oxygenated monoterpenes, six sesquiterpenes, and two oxygenated sesquiterpenes (Table 17). Amongst these compounds, cuminaldehyde (36.3 %) appeared to be the major component, followed by cuminic alcohol (16.9 %), γ-terpinene (11.1 %), safranal (10.9 %), p-cymene (9.9 %) and β-pinene (7.8 %). The list of essential oil compositions has been shown in Table 17.

### 4.1.3 *Fortunella japonica*

#### 4.1.3.1 Leaves

The essential oil from leaves of *Fortunella japonica* was obtained by hydrodistillation. The oil yield was found to be 0.35 % (v/w) of fresh weight. Seventeen compounds was analysed by GC-MS and identified as thirteen monoterpenes and three oxygenated monoterpenes (Table 18). Amongst these compounds, β-pinene (47.44 %) appeared to be the major component, followed by

d-limonene (10.24 %), linalool (9.79 %), trans-ocimene (7.56 %), and α-pinene (7.41 %). The list of essential oil compositions has been shown in Table 18.

#### 4.1.3.2 Peels

The essential oil from peels of *Fortunella japonica* was obtained by hydrodistillation. The oil yield was found to be 0.50 % (v/w) of fresh weight. Nineteen compounds was analysed by GC-MS and identified as ten monoterpenes, five oxygenated monoterpenes, and four sesquiterpenes (Table 19). Amongst these compounds, d-limonene (87.07 %) appeared to be the major component, followed by linalool (1.44 %), myrcene (1.32 %), and geranyl acetate (1.12 %). The list of essential oil compositions has been shown in Table 19.

### 4.1.4 *Pogostemon cablin*

The essential oil from *Pogostemon cablin* was obtained by fresh leaves hydrodistillation. The oil yield was found to be 0.30 % (v/w) of fresh weight. Twenty two compounds was analysed by GC-MS and identified as eighteen sesquiterpenes, and three oxygenated sesquiterpenes. Among of these, patchouli alcohol (60.30 %) appeared to be the major component, and followed by germacrene A (11.73 %). The list of essential oil compositions has been shown in Table 20.

Table 16 Essential oil constituents of *Artemisia vulgaris* var. *indica* obtained by fresh leaves hydrodistillation

| Compound | Kovat's Index | % Area |
|---|---|---|
| **Monoterpenes** | | |
| santolina triene | 0908 | 0.15 |
| sabinene | 0976 | 0.21 |
| α-phellandrene | 1005 | 0.29 |
| δ-2-carene | 1001 | 0.26 |
| *o*-cymene | 1022 | 1.94 |
| γ-terpinene | 1062 | 0.14 |
| **Oxygenated monoterpenes** | | |
| 1,8-cineole | 1033 | 2.07 |
| cis-chrysanthenol | 1162 | 0.46 |
| chrysanthenone | 1123 | 6.65 |
| 4-terpineol | 1177 | 0.51 |
| α-terpineol | 1189 | 0.31 |
| cis-chrysanthenyl acetate | 1262 | 0.37 |
| bornyl acetate | 1285 | 0.09 |
| **Sesquiterpenes** | | |
| α-copaene | 1376 | t |
| 9-epi-β-caryophyllene | 1467 | 3.80 |
| α-humulene | 1454 | 0.98 |
| Germacrenes D | 1480 | 1.92 |
| **Oxygenated sesquiterpenes** | | |
| nordavanone | 1229 | 0.16 |
| cis-threo-davanafuran | 1414 | 0.12 |
| artedouglasia oxide A | 1535 | 0.24 |
| davanone | 1586 | 71.56 |
| juniper camphor | 1691 | 1.63 |
| α-bisabolol acetate | 1796 | 0.55 |
| **Others** | | |
| (*Z*)-3-hexenol | 0857 | 0.43 |
| unidentified 1 | - | 1.00 |
| 3-octanone | 0986 | 0.21 |
| 3-octanol | 0993 | 0.27 |
| unidentified 2 | - | 2.86 |
| unidentified 3 | - | 0.43 |
| unidentified 4 | - | 0.22 |

t = trace (less than 0.01)

Table 17 Essential oil constituents of *Cuminum cyminum* obtained by fruit hydrodistillation

| Compound | Kovat's Index | % Area |
|---|---|---|
| **Monoterpenes** | | |
| α-thujene | 0931 | 0.2 |
| α-pinene | 0939 | 0.5 |
| β-pinene | 0980 | 7.8 |
| myrcene | 0991 | 0.6 |
| α-phellandrene | 1005 | 0.1 |
| δ-3-carene | 1011 | 0.1 |
| α-terpinene | 1018 | 0.1 |
| p-cymene | 1026 | 9.9 |
| d-limonene | 1031 | 0.5 |
| γ-terpinene | 1062 | 11.1 |
| terpinolene | 1088 | 0.1 |
| **Oxygenated monoterpene** | | |
| 1,8-cineol | 1033 | 0.3 |
| cis-sabinene hydrate | 1068 | t |
| linalool | 1098 | t |
| cis-p-menth-2-en-1-ol | 1121 | t |
| trans-pinocarveol | 1139 | 0.1 |
| pulegone | 1237 | 0.1 |
| pinocarvone | 1162 | t |
| 4-terpineol | 1177 | 0.6 |
| isopulegol | 1146 | 0.4 |
| myrtenol | 1194 | 0.1 |
| cuminaldehyde | 1239 | 36.3 |
| phellandral | - | 0.2 |
| safranal | - | 10.9 |
| cuminic alcohol | - | 16.9 |
| p-mentha-1,4-en-7-ol | - | 0.3 |
| **Sesquiterpenes** | | |
| β-caryophellene | 1418 | 0.1 |
| trans-β-farnesene | 1458 | 0.4 |
| germacrene D | 1480 | 0.1 |
| α-acoradiene | 1463 | 0.1 |
| β-chamigrene | 1475 | t |
| β-bisabolene | 1509 | 0.1 |
| **Oxygenated sesquiterpenes** | | |
| caryophyllene oxide | 1581 | t |
| carotol | 1594 | 0.1 |

t = trace (less than 0.01)

Table 18 Essential oil constituents of *Fortunella japonica* obtained by fresh leaves hydrodistillation

| Compound | Kovat's Index | % Area |
|---|---|---|
| **Monoterpenes** | | |
| α-thujene | 0931 | 0.91 |
| α-pinene | 0931 | 7.41 |
| camphene | 0953 | 3.59 |
| β-pinene | 0980 | 47.44 |
| myrcene | 0991 | 3.16 |
| α-phellandrene | 1005 | 0.95 |
| δ-3-carene | 1011 | 0.77 |
| α-terpinene | 1018 | 1.82 |
| d-limonene | 1031 | 10.24 |
| cis-ocimene | 1040 | 0.91 |
| trans-ocimene | 1050 | 7.56 |
| γ-terpinene | 1062 | 2.56 |
| terpinolene | 1088 | 1.50 |
| **Oxygenated monoterpenes** | | |
| linalool | 1098 | 9.79 |
| 4-terpineol | 1177 | 0.57 |
| α-terpineol | 1189 | 0.44 |
| **Others** | | |
| n-decanal | 1204 | 0.40 |

Table 19 Essential oil constituents of *Fortunella japonica* obtained by peel hydrodistillation

| Compound | Kovat's Index | % Area |
|---|---|---|
| **Monoterpenes** | | |
| α-pinene | 0931 | 0.83 |
| δ-3-carene | 1011 | t |
| myrcene | 0991 | 1.32 |
| α-terpinene | 1018 | t |
| d-limonene | 1031 | 87.07 |
| β-phellandrene | 1031 | 0.19 |
| γ-terpinene | 1062 | 0.03 |
| trans-ocimene | 1050 | 0.08 |
| p-cymeme | 1026 | t |
| terpinolene | 1088 | 0.04 |
| **Oxygenated monoterpenes** | | |
| linalool | 1098 | 1.44 |
| 4-terpineol | 1177 | 0.27 |
| α-terpineol | 1189 | 0.13 |
| geranyl acetate | 1383 | 1.12 |
| geraniol | 1255 | 0.02 |
| **Sesquiterpenes** | | |
| germacrene D | 1480 | 0.81 |
| β-elemene | 1391 | 0.04 |
| α-copaene | 1376 | 0.02 |
| α-humulene | 1454 | 0.06 |

t = trace (less than 0.01)

Table 20 Essential oil constituents of *Pogostemon cablin* obtained by fresh leaves hydrodistillation

| Compound | Kovat's Index | % Area |
|---|---|---|
| **Sesquiterpenes** | | |
| δ-elemene | 1339 | t |
| β-patchoulene | 1380 | t |
| β-elemene | 1391 | 0.33 |
| cis-thujopsene | 1429 | 0.25 |
| trans-caryophyllene | 1418 | 2.24 |
| α-guaiene | 1439 | 7.22 |
| γ-patchoulene | 1441 | 3.89 |
| α-humulene | 1454 | 0.48 |
| α-patchoulene | 1456 | 2.27 |
| seychellene | 1460 | 0.98 |
| valencene | 1491 | 0.85 |
| germacrene D | 1480 | 0.15 |
| β-selinene | 1485 | t |
| α-selinene | 1494 | 0.23 |
| viridiflorene | 1493 | 1.91 |
| germacrene A | 1503 | 11.73 |
| α-bulnesene | 1505 | 0.86 |
| 7-epi-α-selinene | 1517 | 0.17 |
| **Oxygenated sesquiterpenes** | | |
| longipinanol | 1566 | t |
| globulol | 1583 | 4.62 |
| patchouli alcohol | 1659 | 60.30 |
| **Others** | | |
| 1-octen-3-ol | 0978 | 0.20 |
| unidentified | - | 1.19 |

t = trace (less than 0.01)

**4.2 Determination of germination and growth of seedlings**

Fruits of *Cuminum cyminum* and seeds of *Fortunella japonica* were surface sterilised by same method as shown in Table 21. Each explant was dipped in 70% ethanol, 1 min followed by 30% $H_2O_2$, 5 min. Then, they were cleaned 3 times in distilled water before germination.

Table 21 Surface sterilisation fruits of *Cuminum cyminum* and seeds of *Fortunella japonica*

| Species | Explants | Surface sterilisation |
|---|---|---|
| *Cuminum cyminum* | Fruits | 70 % Ethanol, 1 min ↓ 30% $H_2O_2$, 5 min |
| *Fortunella japonica* | Seeds | |

After surface sterilisation, aseptic plant materials were aseptically transferred to pre-sterilised glass petri dishes, each one containing two pieces of Whatman No.1 filter paper and containing about 20 ml distilled water, and incubated in the dark at 25 ± 2 °C. The germination of seedling was depended on the sterilisation time period. No germination was observed when sterilisation time was more than 5 minutes. Table 22 shows the germination of individual seeds. After they had germinated, they were put in 12 hour light/dark intervals to develop strong seedlings.

Table 22 Germination of *Cuminum cyminum* and *Fortunella japonica*

| Species | Result |
|---|---|
| *Cuminum cyminum* | 3* |
| *Fortunella japonica* | 4 |

* 4 = good germination, 3 = moderate germination, 2 = slight germination

### 4.3 Surface sterilisation of leaves of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin*

Leaf explants of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin* were surface sterilised by following methods as shown in Table 23 prior to use in callus initiation.

Table 23 Surface sterilisation leaves of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin*

| Species | Explants | Surface sterilisation |
|---|---|---|
| *Artemisia vulgaris* var. *indica* | Leaves | Surface sterilising agent*,<br>1 hr<br>↓<br>7% $H_2O_2$, 15 min<br>↓<br>5% $H_2O_2$, 7 min |
| *Pogostemon cablin* | Leaves | 5 % Clorox, 5 min |

* The compositions of surface sterilising agent were described in part C of Appendix

### 4.4 Growth and appearance of callus cultures

Callus cultures of the individual plants were initiated from the seedlings or sterilised leaf explants. Table 24 shows list details of appearances of callus cultures and growth of the individual species. It was shown that *Fortunella japonica* callus cultures grew fastest (++++). They were subcultured every 14-21 days on average. These callus cultures appeared mainly cream, pale green and green in colour, with a friable and crumbly appearance. The other calli grew well also (+++), and they were subcultured every 21-28 days on average. Table 24 has shown lists details of appearances and growth of individual callus cultures and Fig. 22-25 have shown appearance of individual callus cultures.

Table 24 Appearance and callus growth of individual species

| Species | Appearance | Growth |
|---|---|---|
| *Artemisia vulgaris* var. *indica* | Yellowish-brown, compact | +++ |
| *Cuminum cyminum*, | Pale green, green, friable | +++ |
| *Fortunella japonica* | Pale green, yellowish-green, friable | ++++ |
| *Pogostemon cablin* | Greenish-brown, brown, compact | +++ |

++++ = profuse growth +++ = good growth ++ = moderate growth

\+ = slight growth - = no growth

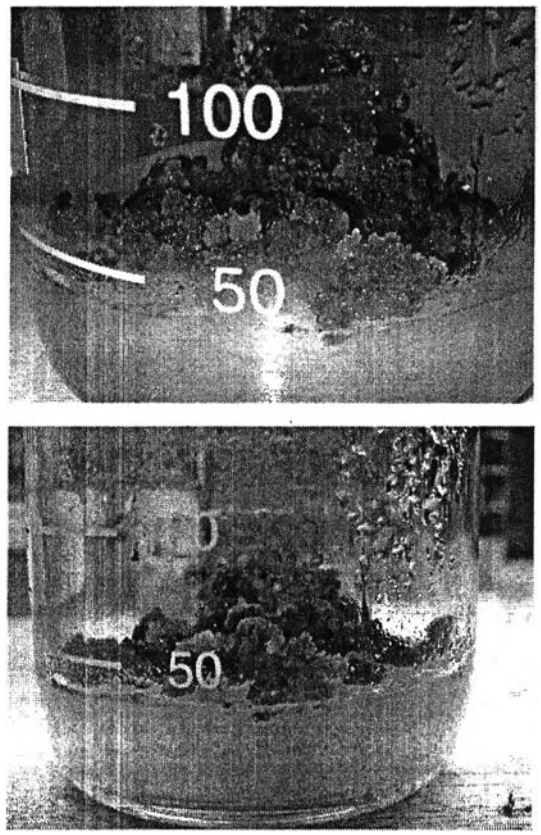



Figure 22 Callus cultures of *Artemisia vulgaris* var. *indica*

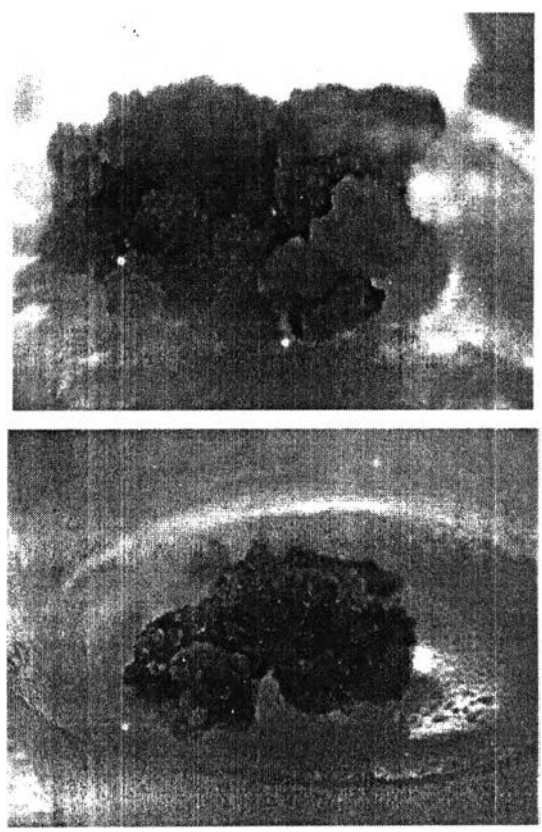


Figure 23 Callus cultures of *Cuminum cyminum*

### 4.5 Effect of plant growth regulators on callus formation and appearance

Various types and concentrations of plant growth regulators applied in MS media were varied to study their effects on callus formation of the investigated plants. Most plant species had been grown in MS media containing 1 mg/l 2,4 dichlorophenoxy-acetic acid and 0.1 mg/l kinetin, excepted for *Pogostemon cablin* had to be grown in 0.5 mg/l napthaleneacetic acid and 1 mg/l 6-benzyladenine. Plant growth regulators used for maintenance callus cultures has been listed in Table 25.

Table 25 List of plant growth regulators used for maintenance callus cultures

| Species | Plant growth regulators |
|---|---|
| *Artemisia vulgaris* var. *indica* | 1 mg/l 2,4-D + 0.1 mg/l Kn |
| *Cuminum cyminum* | 1 mg/l 2,4-D + 0.1 mg/l Kn |
| *Fortunella japonica* | 1 mg/l 2,4-D + 0.1 mg/l Kn |
| *Pogostemon cablin* | 0.5 mg/l NAA + 1 mg/l BA |

### 4.6 Effect of light on callus formation and appearance

Callus cultures of various plants were incubated at the temperature of 25 ± 2 °C under different light conditions; 24-h light, 12-h light/12-h dark, and 24-h dark. Most plant species had been incubated in 24 h light condition, excepted for *Pogostemon cablin* had to be incubated in 24 h dark condition as shown in Table 26.

Table 26 Light condition used for maintenance callus cultures

| Species | Light conditions |
|---|---|
| *Artemisia vulgaris* var. *indica* | Light, 24 h |
| *Cuminum cyminum* | Light, 24 h |
| *Fortunella japonica* | Light, 24 h |
| *Pogostemon cablin* | Dark, 24 h |

### 4.7 Growth and appearance of cell suspension cultures

Cell suspension cultures were derived from healthy fourth generation callus cultures. Each callus cultures were aseptically transferred in liquid media (the composition is same as using for maintenance callus cultures but without any agar). Fine suspension cultures were observed for *Fortunella japonica* (Fig.28)

Cell suspension cultures of *Fortunalla japonica* are the best growing. They grew as yellow-coloured suspensions, and were subcultured in 14-21 days intervals.

Cell suspension cultures of *Cuminum cyminum* appeared green in colour, and the biomass was less than that from *Fortunella japonica*. They were subcultured in 21-28 days intervals.

Cell suspension cultures of *Artemisia vulgaris* var. *indica* and *Pogostemon cablin* appeared brown in colour, and were subcubculture in 21-28 days intervals.

Table 27 has shown lists details of appearances and growth of individual cell suspension cultures and Fig. 26-29 have shown appearance of individual cultures.

Table 27 Appearance and growth of suspension cultures of individual species

| Species | Appearance | Growth |
|---|---|---|
| *Artemisia vulgaris* var. *indica* | Pale brown, brown | +++ |
| *Cuminum cyminum*, | Green, greenish-brown | +++ |
| *Fortunella japonica* | Yellow, dark yellow | ++++ |
| *Pogostemon cablin* | Brown, dark brown | ++ |

++++ = profuse growth +++ = good growth ++ = moderate growth

\+ = slight growth - = no growth

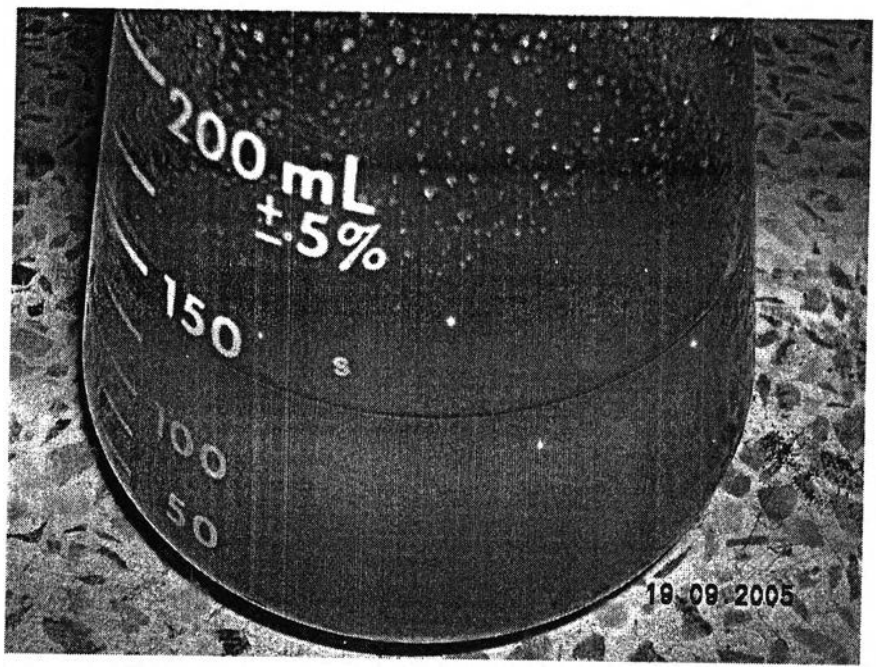



Figure 26 Suspension cultures of *Artemisia vulgaris* var. *indica*

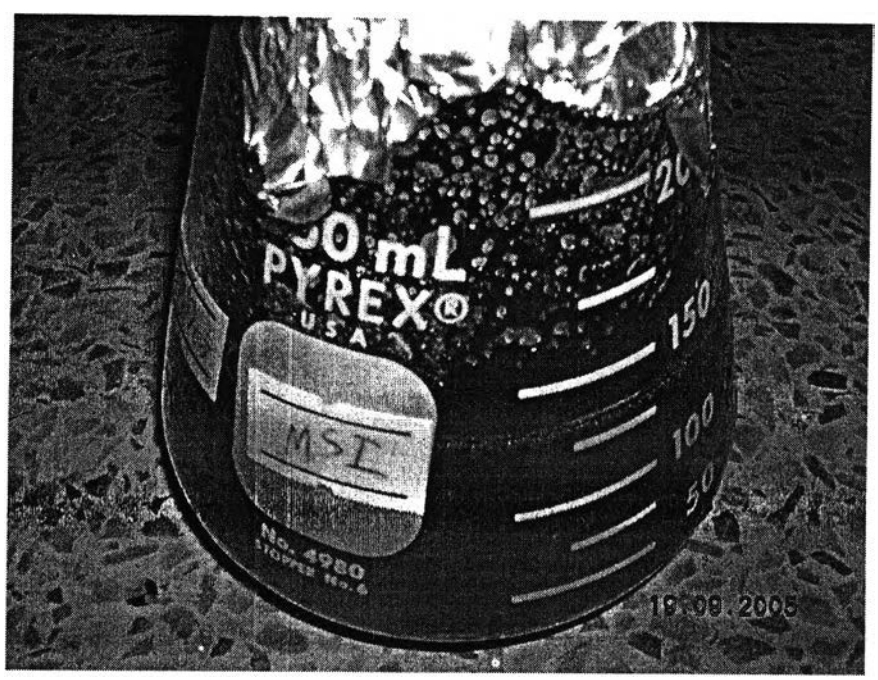



Figure 27 Suspension cultures of *Cuminum cyminum*

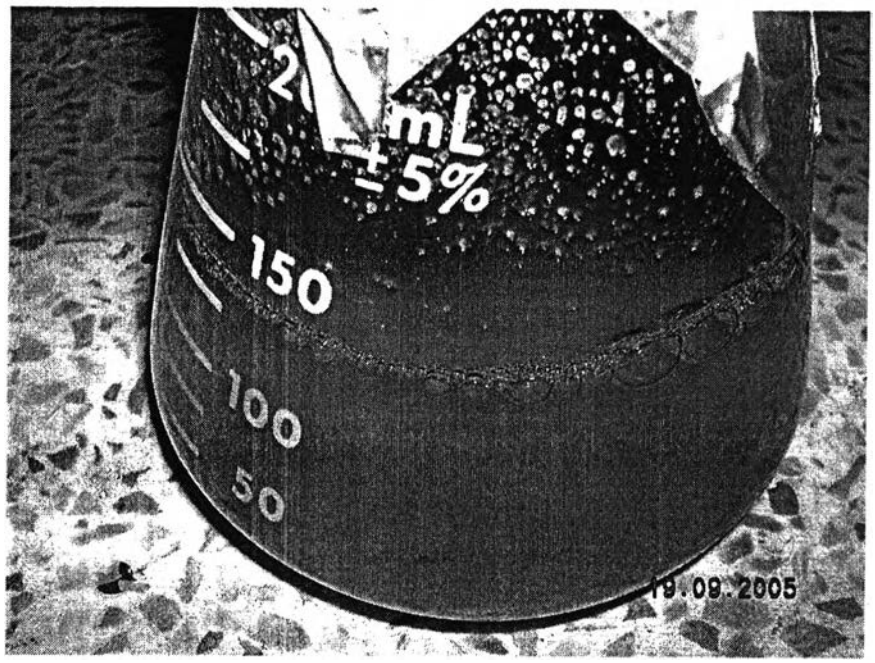



Figure 28 Suspension cultures of *Fortunella japonica*

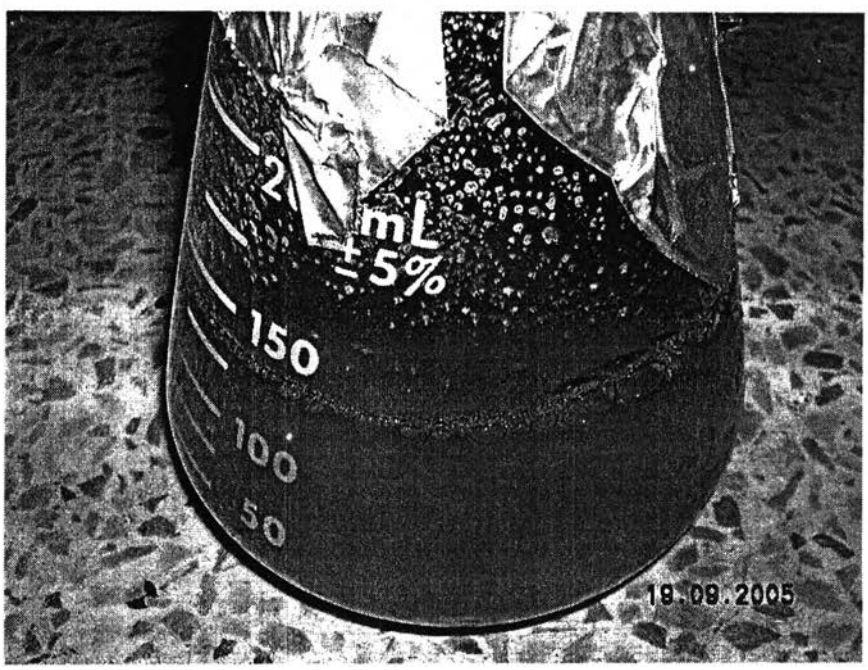



Figure 29 Suspensicn cultures of *Pogostemon cablin*

## 4.8 Identification the essential oil constituents produced by plant cell cultures by Gas Chromatography-Mass Spectrometry (GC-MS)

To identify the essential oil constituents produced by callus and cell suspension cultures, they were extracted and analysed by the methods described in 3.14. Table 28 shows essential oil constituents produced by individual plant cell cultures obtained by dichloromethane extraction.

Table 28 Essential oil constituents of *Artemisia vulgaris* var. *indica* callus cultures obtained by dichloromethane extraction

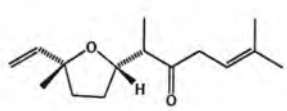
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Davanone | 20.5 |  | 1586 | 41, 55, 69, 81, 93, 111, 125, 139, 153, 180, 236 |

Table 29 Essential oil constituents of *Artemisia vulgaris* var. *indica* cell suspension cultures obtained by dichloromethane extraction

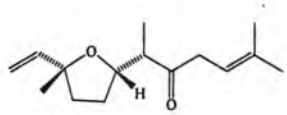
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Davanone | 21.5 |  | 1586 | 41, 55, 69, 81, 93, 111, 125, 139, 153, 180, 236 |

Table 30 Essential oil constituents of *Cuminum cyminum* callus cultures obtained by dichloromethane extraction

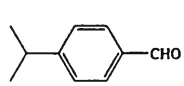
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Cumin aldehyde | 28.35 |  | 1239 | 41, 51, 63, 77, 91, 105, 115, 119, 133, 148 |

Table 31 Essential oil constituents of *Cuminum cyminum* cell suspension cultures obtained by dichloromethane extraction

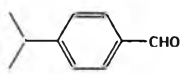
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Cumin aldehyde | 26.77 |  | 1239 | 41, 51, 63, 77, 91, 105, 115, 119, 133, 148 |

Table 32 Essential oil constituents of *Fortunella japonica* callus cultures obtained by dichloromethane extraction

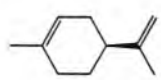
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| d-Limonene | 25.5 |  | 1031 | 41, 53, 67, 79, 93, 107, 121, 136 |

Table 33 Essential oil constituents of *Fortunella japonica* cell suspension cultures obtained by dichloromethane extraction

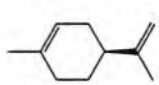
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| d-Limonene | 22.5 |  | 1031 | 41, 53, 67, 79, 93, 107, 121, 136 |

Table 34 Essential oil constituents of *Pogostemon cablin* callus cultures obtained by dichloromethane extraction

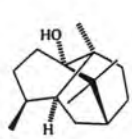
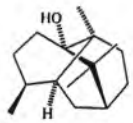
| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Patchouli alcohol | 19.85 |  | 1659 | 41, 55, 67, 81, 95, 109, 125, 138, 161, 189, 205, 222 |

Table 35 Essential oil constituents of *Pogostemon cablin* cell suspension cultures obtained by dichloromethane extraction

| Compounds | Yield (ppm) | Structure | Kovat's index | MS data |
|---|---|---|---|---|
| Patchouli alcohol | 19.5 |  | 1659 | 41, 55, 67, 81, 95, 109, 125, 138, 161, 189, 205, 222 |

The results shown in Table 28-35, have revealed that the major constituents of the essential oil can be produced in callus and cell suspension cultures of individual plants. However, some minor constituents can be found in very low level in each cell extracts, and data not shown in each Table.

**4.9 Time-course studies of volatile constituent content during the growth cycle of plant cell cultures**

In attempt to increase level of the major constituents in each cell culture, the time-course of each compound during the growth cycles had to be studied. Levels of major constituents and growth rate of callus and suspension cultures were determined and these are shown in Table 36-43 and Fig. 30-37.

Table 36 Fresh weight, dry weight and davanone content in *Artemisia vulgaris* var. *indica* callus cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Davanone content (ppm) |
|---|---|---|---|
| 0 | 3.8 | 0.418 | 1.95 |
| 7 | 4.5 | 0.54 | 2.45 |
| 14 | 8.5 | 1.02 | 8.55 |
| 21 | 12.5 | 1.5 | 20.5 |
| 28 | 14.2 | 1.56 | 13.09 |
| 35 | 15.6 | 1.71 | 12.25 |

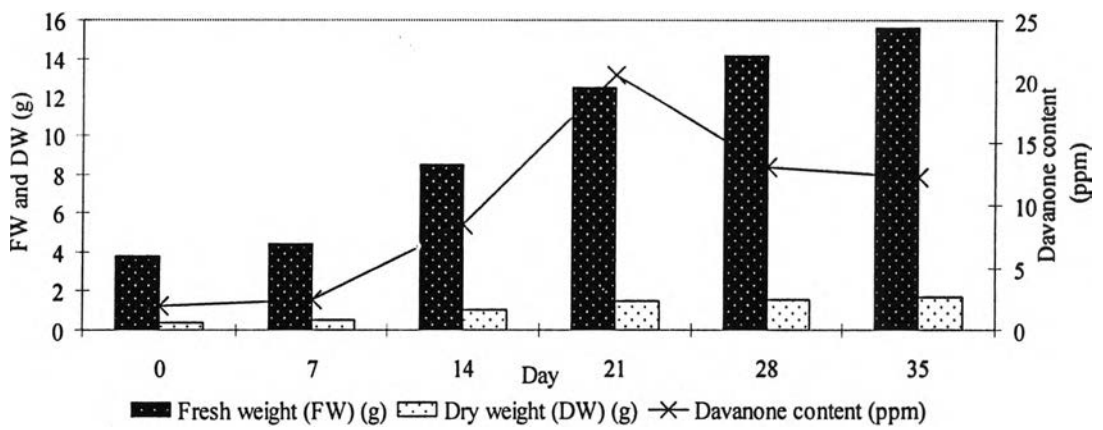



Figure 30 Time-course of growth and the formation of davanone in *Artemisia vulgaris* var. *indica* callus cultures (SD<5%, n=3)

Table 37 Fresh weight, dry weight and davanone content in *Artemisia vulgaris* var. *indica* cell suspension cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Davanone content (ppm) |
|---|---|---|---|
| 0 | 1.45 | 0.17 | 2.85 |
| 7 | 2.98 | 0.36 | 3.55 |
| 14 | 8.33 | 0.92 | 9.45 |
| 21 | 12.65 | 1.4 | 21.5 |
| 28 | 13.2 | 1.45 | 15.09 |

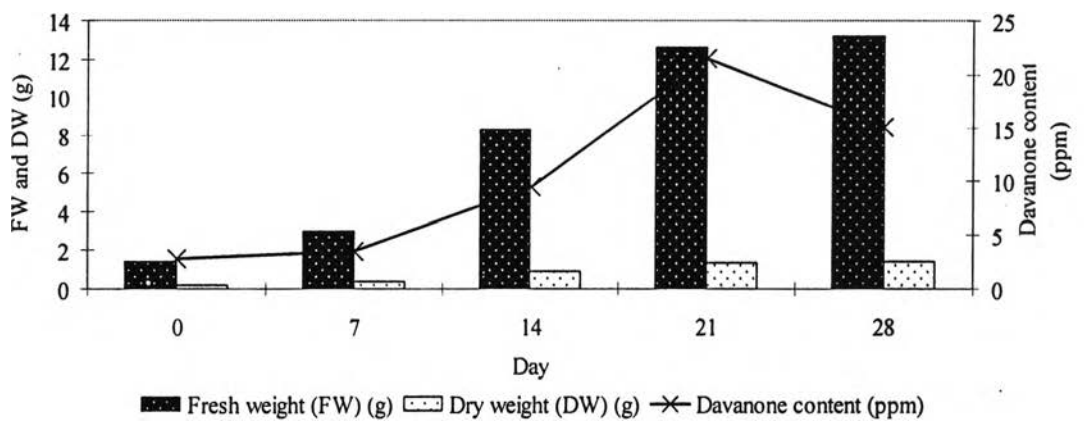



Figure 31 Time-course of growth and the formation of davanone in *Artemisia vulgaris* var. *indica* cell suspension cultures (SD<5%, n=3)

Table 38 Fresh weight, dry weight and cuminaldehyde content in *Cuminum cyminum* callus cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Cuminaldehyde content (ppm) |
|---|---|---|---|
| 0 | 3.2 | 0.34 | 1.55 |
| 7 | 3.7 | 0.4 | 2.75 |
| 14 | 4.9 | 0.57 | 8.95 |
| 21 | 6.1 | 0.63 | 18.95 |
| 28 | 8.1 | 0.81 | 26.45 |
| 35 | 9.5 | 0.99 | 28.35 |
| 42 | 9.8 | 1.03 | 19.45 |

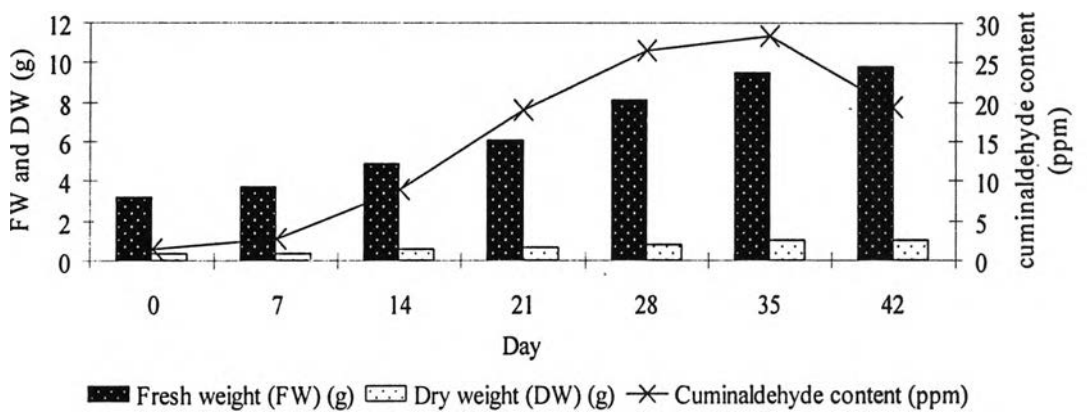



Figure 32 Time-course of growth and the formation of cuminaldehyde in *Cuminum cyminum* callus cultures (SD<5%, n=3)

Table 39 Fresh weight, dry weight and cuminaldehyde content in *Cuminum cyminum* cell suspension cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Cuminaldehyde content (ppm) |
|---|---|---|---|
| 0 | 1.07 | 0.16 | 1.43 |
| 7 | 2.56 | 0.36 | 2.55 |
| 14 | 7.5 | 1.24 | 6.45 |
| 21 | 9.23 | 1.48 | 26.77 |
| 28 | 9.44 | 1.51 | 19.56 |

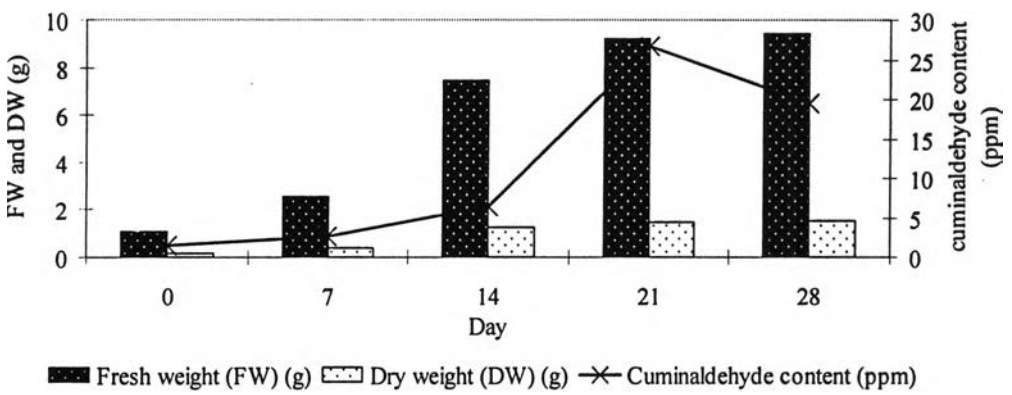



Figure 33 Time-course of growth and the formation of cuminaldehyde in *Cuminum cyminum* cell suspension cultures (SD<5%, n=3)

Table 40 Fresh weight, dry weight and d-limonene content in *Fortunella japonica* callus cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | d-Limonene content (ppm) |
|---|---|---|---|
| 0 | 3.5 | 0.385 | 1.95 |
| 7 | 3.9 | 0.468 | 3.25 |
| 14 | 6.5 | 1.17 | 4.75 |
| 21 | 9.5 | 1.81 | 17.65 |
| 28 | 11.7 | 2.36 | 25.5 |
| 35 | 12.3 | 2.45 | 21.75 |

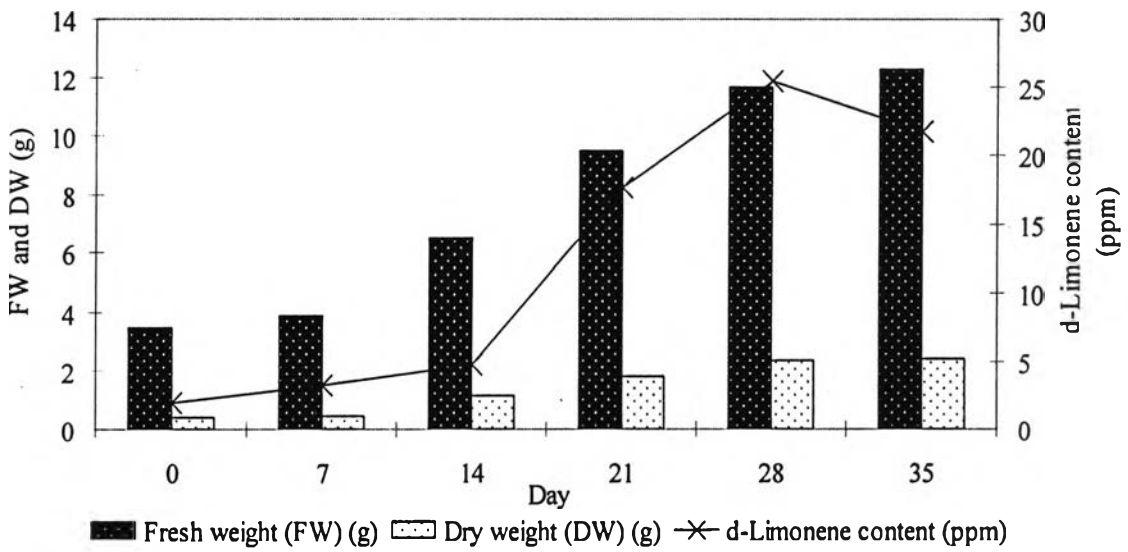



Figure 34 Time-course of growth and the formation of d-limonene in *Fortunella japonica* callus cultures (SD<5%, n=3)

Table 41 Fresh weight, dry weight and d-limonene content in *Fortunella japonica* cell suspension cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | d-Limonene content (ppm) |
|---|---|---|---|
| 0 | 1.36 | 0.16 | 1.85 |
| 7 | 2.76 | 0.34 | 2.25 |
| 14 | 7.81 | 1.47 | 5.35 |
| 21 | 11.5 | 2.15 | 22.5 |
| 28 | 12.2 | 2.45 | 18.75 |

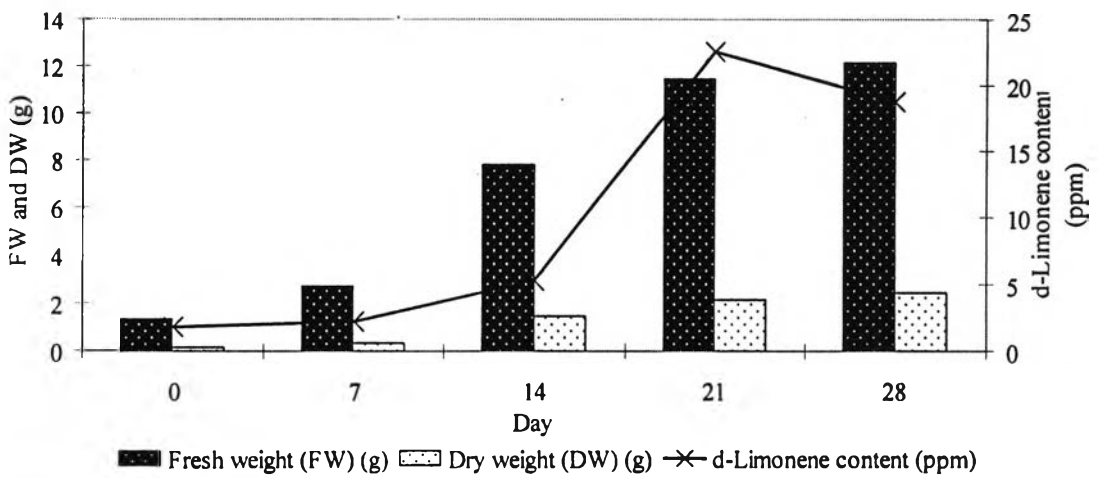



Figure 35 Time-course of growth and the formation of d-limonene in *Fortunella japonica* cell suspension cultures (SD<5%, n=3)

Table 42 Fresh weight, dry weight and patchouli alcohol content in *Pogostemon cablin* callus cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Patchouli alcohol content (ppm) |
|---|---|---|---|
| 0 | 2.8 | 0.31 | 0.65 |
| 7 | 2.9 | 0.34 | 2.15 |
| 14 | 3.2 | 0.37 | 5.5 |
| 21 | 4.1 | 0.54 | 12.55 |
| 28 | 7.5 | 1.05 | 14.8 |
| 35 | 8.5 | 1.1 | 19.85 |
| 42 | 8.9 | 1.15 | 16.75 |

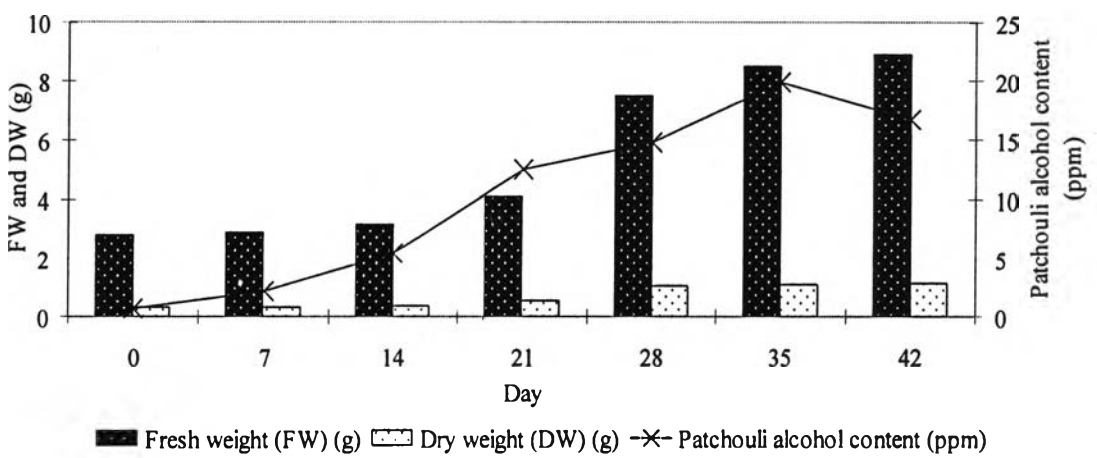



Figure 36 Time-course of growth and the formation of patchouli alcohol in *Pogostemon cablin* callus cultures (SD<5%, n=3)

Table 43 Fresh weight, dry weight and patchouli alcohol content in *Pogostemon cablin* cell suspension cultures (SD<5%, n=3)

| Day | Fresh weight (FW) (g) | Dry weight (DW) (g) | Patchouli alcohol content (ppm) |
|---|---|---|---|
| 0 | 1.12 | 0.14 | 0.85 |
| 7 | 2.47 | 0.37 | 1.35 |
| 14 | 3.89 | 0.58 | 4.5 |
| 21 | 6.62 | 1.19 | 19.5 |
| 28 | 7.74 | 1.39 | 15.55 |

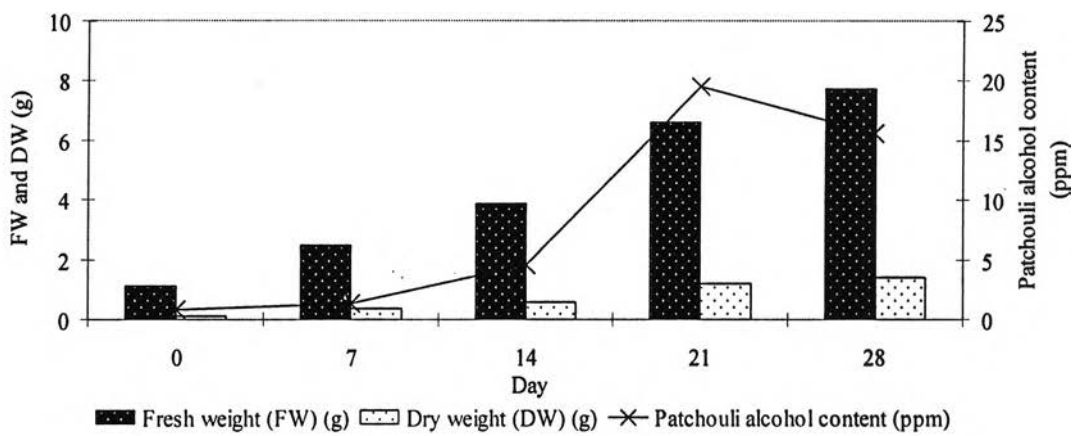



Figure 37 Time-course of growth and the formation of patchouli alcohol in *Pogostemon cablin* cell suspension cultures (SD<5%, n=3)

**4.10 Shoot regeneration (organogenesis)**

Two auxins (NAA and 2,4-D) and three cytokinins (BA, Kn, and TDZ) were applied to culture media at the concentration of 0.5, 1, 2, 3, and 5 mg/l. The effect of various plant growth regulators on shoot formation was measured by investigation of individual growth of various plants, fresh weight determination, and dry weight determination.

After the fourth generation, callus cultures of *Cuminum cyminum* were subcultured to new culture media and successfully formed shoot cultures on MS media containing 1 mg/l NAA and 5 mg/l BA.

*Cuminum cyminum* callus cultures
MS containing 1 mg/l 2,4-D and 0.1 mg/l Kn
↓
MS containing 1 mg/l NAA and 5 mg/l BA
↓
*Cuminum cyminum* shoot cultures

Meanwhile, callus cultures of *Fortunella japonica* could not form shoot cultures in MS media containing any concentration of NAA or BA. However, they had been subcultured with various concentrations of TDZ (0.5-5 mg/l) in one generation, and after that they were subcultured to MS media containing low level of auxin (0.1 mg/l NAA). Finally, callus cultures of *Fortunella japonica* successfully formed shoot cultures. The suitable TDZ concentration for shoot regeneration of this plant cultures was 2 mg/l.

*Fortunella japonica* callus cultures
MS containing 1 mg/l 2,4-D and 0.1 mg/l Kn
↓
Various TDZ concentrations (0.5, 1, 2, 3, and 5 mg/l)
↓
0.1 mg/l NAA
↓
*Fortunella japonica* shoot cultures

## 4.11 Methods for improving chemical constituents of essential oil produced by plant cell cultures

### 4.11.1 Study on feeding precursor and biotransformation

#### 4.11.1.1 Effect of substrate concentration on biotransformation

Since monoterpenes are known to be toxic to plant cells, therefore it is important to find a suitable substrate concentration which can safely be fed to suspension cultures without affecting cell growth. Various geraniol concentrations (50, 100, 200, and 400 ppm) were administered to suspension cultures of *Fortunella japonica* (at the age of 14 days old after subculture; early stationary phase) and sampling was collected every 3 hr until 24 hr after feeding. Control experiment was cell suspension cultures which had not had added any concentrations of geraniol.

As the results shown in Fig. 38 geraniol concentrations up to 200 ppm appears to be toxic to suspension cultures of *Fortunella japonica* since their growth rate determination (FW and DW) are lower than control experiment, meanwhile 50 and 100 ppm geraniol feeding seem to do not effect cell growth. The results of effect of substrate concentration on biotransformation are shown in Fig. 38.

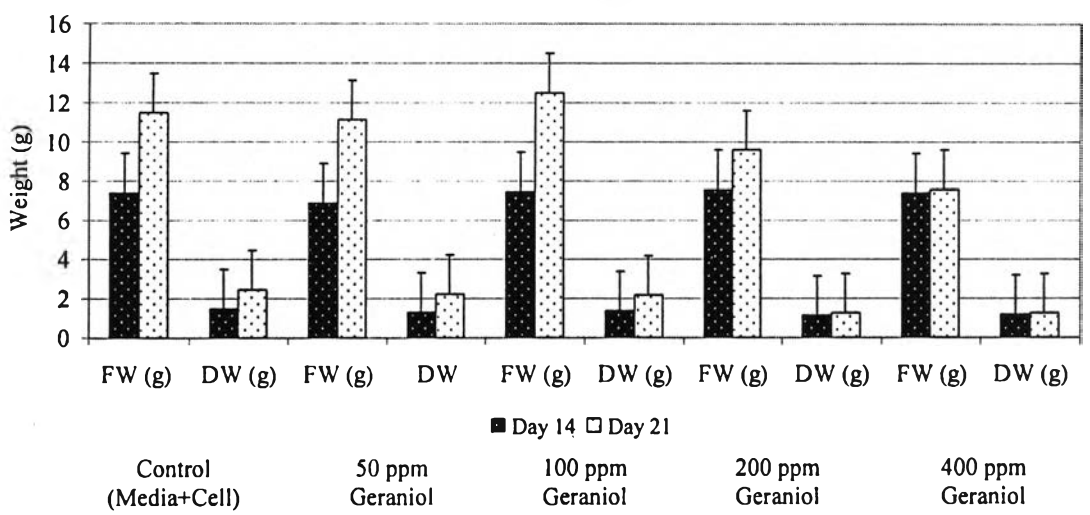



Figure 38 Fresh weight and dry weight of *Fortunella japonica* cell suspension cultures after feeding various concentration of geraniol

### 4.11.1.2 Time-course study of monoterpene feeding to suspension cultures of *Fortunella japonica*

The various concentrations of geraniol (50, 100, 200, and 400 ppm) had been fed in to cell suspension cultures of *Fortunella japonica* and time-course has been shown in Fig. 39. The level of geraniol concentrations decreased rapidly within 6 hr and still retained at very low level in cell cultures until 24 hr. According to the result above in 4.11.1.1 shown 100 ppm geraniol feeding in cell cultures did not effect cell growth, and it is still remained until 24 hr but could be detectable at low level. Since 50 ppm geraniol feeding to cell cultures did not effect cell growth, however, its level was disappeared after feeding 3 hr. Therefore, 100 ppm has been the best concentration feeding to the cell cultures of *Fortunella japonica* and selected to be the suitable concentration for the other terpenoid precursors feeding through out the experiment. The time-course study of geraniol levels after feeding various concentrations in *Fortunella japonica* cell suspension cultures has been shown in Fig. 39.

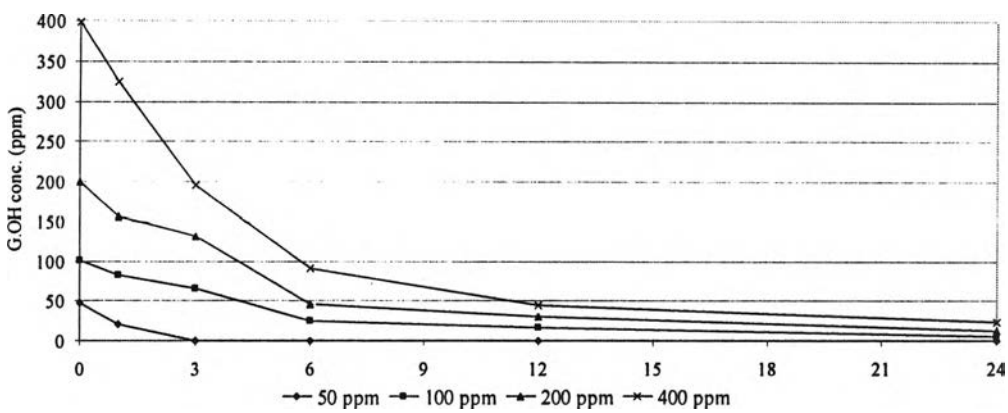



Figure 39 Time-course study of geraniol levels after feeding various concentrations in *Fortunella japonica* cell suspension cultures over 24 hours

### 4.11.1.3 Study of relationship between citral, geraniol, nerol, geranyl acetate, and neryl acetate in cell suspension cultures of *Fortunella japonica*

To study the possible pathway of biotransformation of citral, geraniol, nerol, geranyl acetate, and neryl acetate in cell suspension cultures of *Fortunella japonica*, 100 ppm of these substance were fed into the cells, and then samples were collected every 1 hr until 6 hr. Control experiments were cell suspension cultures which were not added any substrates, and culture media plus substrate with no cells.

As shown in Fig 40, after feeding citral, the isomer of neral and geranial, it could be biotransformed to their correspondence alcohol, nerol and geraniol, respectively. It had been shown that *Fortunella japonica* cell suspension had got the reducing enzyme. Citral had been decreased and disappeared since 3rd hour after feeding. The highest concentrations of nerol and geraniol had been taken place at 1st hour after feeding and both of them had been decreased. Geranyl acetate could be detected at 3rd hour and then it has been decreased in trace, meanwhile neryl acetate could not be detected at anytimes.

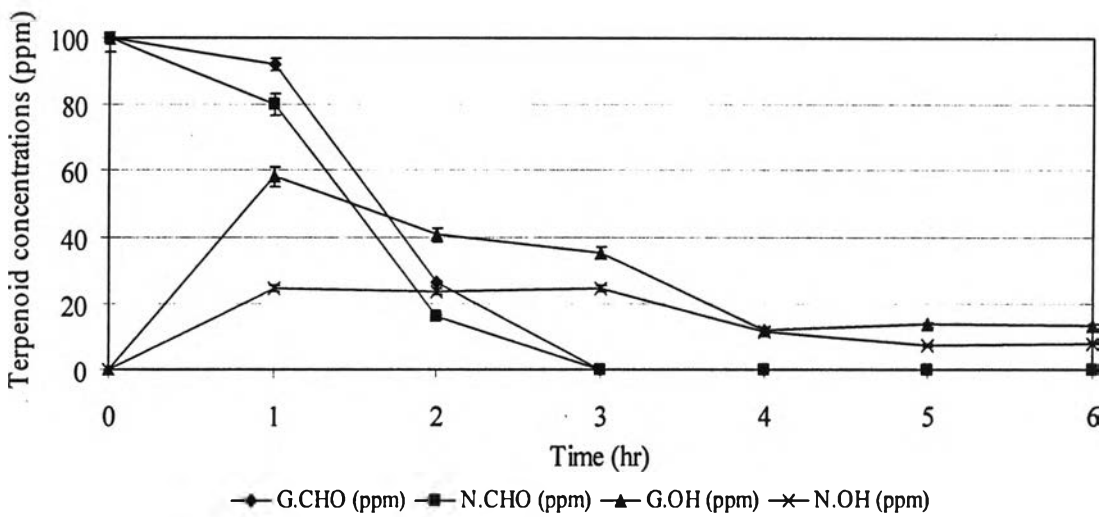



Figure 40 Terpenoid concentrations after feeding 100 ppm citral in *Fortunella japonica* cell suspension cultures

The possible pathway of biotransformation of citral in *Fortunella japonica* is shown in Fig. 41 below.

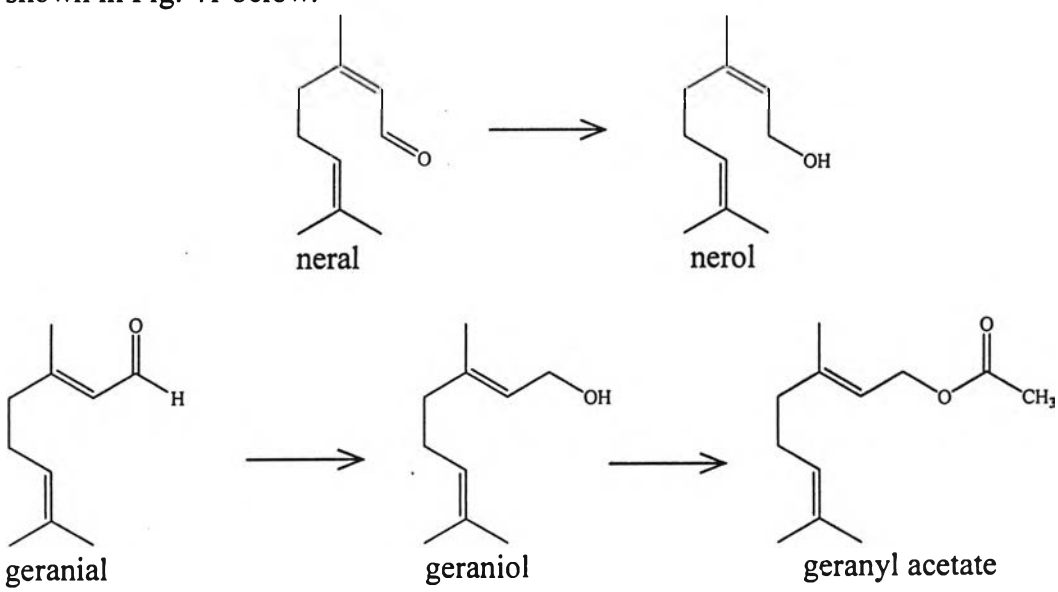



Figure 41 The possible pathway of biotransformation of citral in *Fortunella japonica*

As shown in Fig. 42, after feeding geraniol, it could be biotransformed to nerol, its isomer alcohol. It had been shown that *Fortunella japonica* cell suspension has got the isomerisation enzyme. The highest concentration of nerol had been taken place at 3rd hour after feeding and then both geraniol and nerol had been decreased. Geranyl acetate could be detected in trace since 2nd hour, meanwile neryl acetate can not be detected at anytimes.

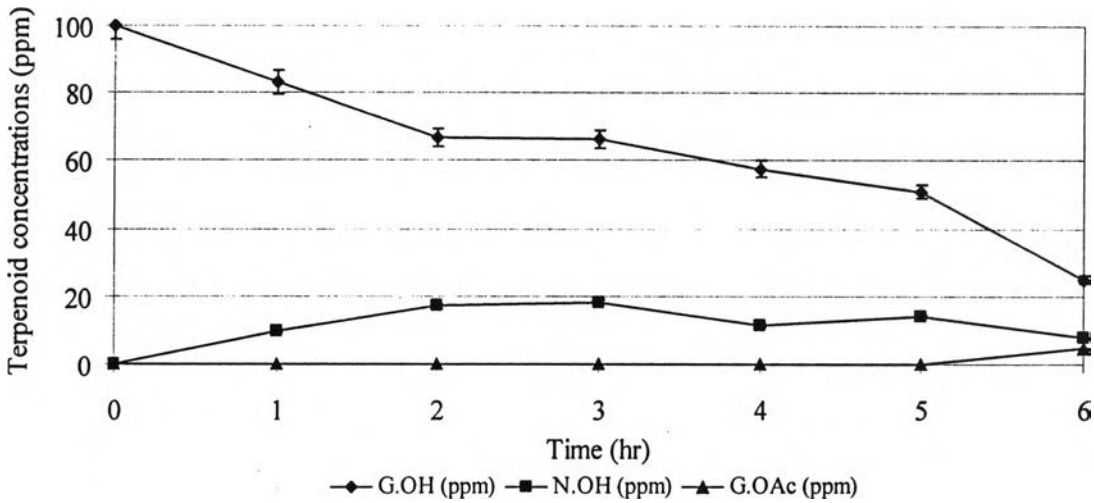
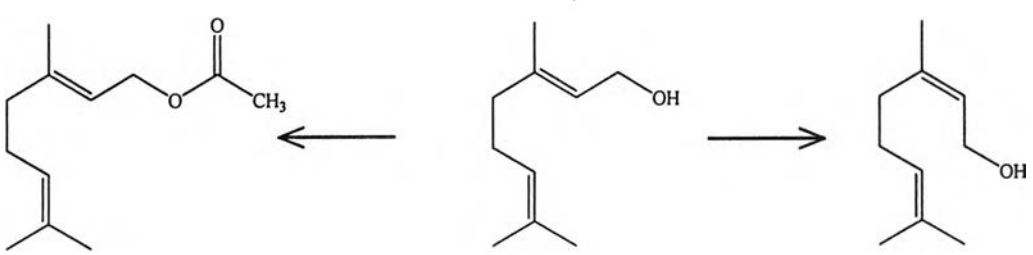



Figure 42 Terpenoid concentrations after feeding 100 ppm geraniol in *Fortunella japonica* cell suspension cultures

The possible pathway of biotransformation of geraniol in *Fortunella japonica* is shown in Fig. 43 below.




geranyl acetate geraniol nerol

Figure 43 The possible pathway of biotransformation of geraniol in *Fortunella japonica*

As shown in Fig. 44, after feeding nerol, it could be biotransformed to geraniol, its isomer alcohol. It has been shown that *Fortunella japonica* cell suspension has got the isomerisation enzyme. The highest concentration of geraniol was taken place at 3rd hour after feeding and then both nerol and geraniol have been decreased. Neryl acetate can be detected since the 2nd hour in the GC trace as well.

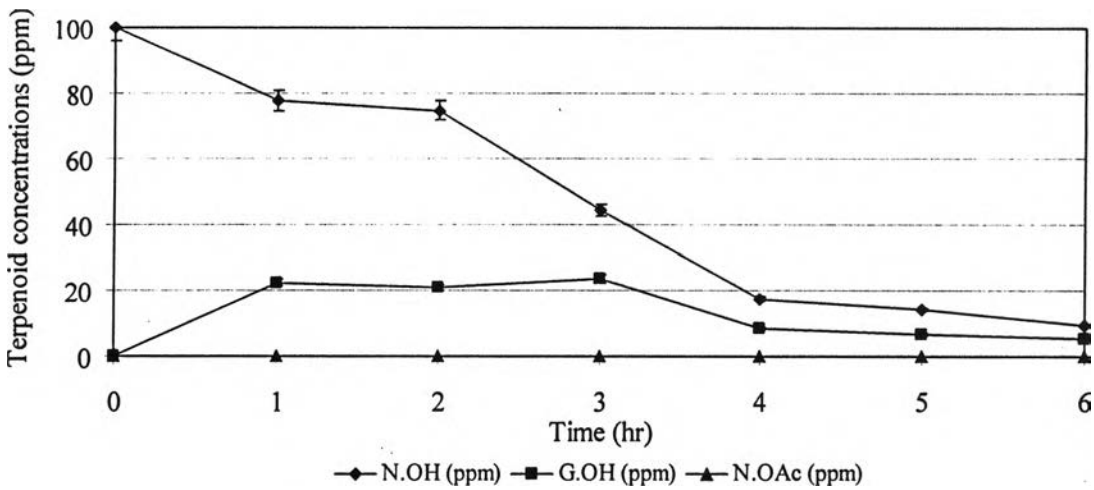
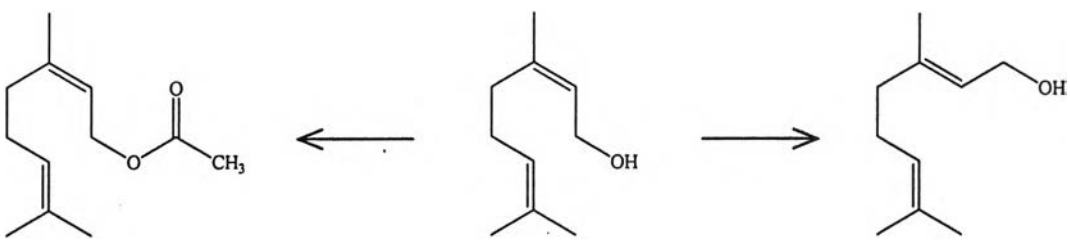



Figure 44 Terpenoid concentrations after feeding 100 ppm nerol in *Fortunella japonica* cell suspension cultures

The possible pathway of biotransformation of nerol is shown in Fig. 45 below.




neryl acetate nerol geraniol

Figure 45 The possible pathway of biotransformation of nerol in *Fortunella japonica*

As shown in Fig. 46, after feeding geranyl acetate, it could be biotransformed to nerol and geraniol. It had been decreased and disappeared since 3rd hour after feeding. The highest concentration of geraniol and nerol were taken place in 1st and 2nd hour after feeding, respectively, and then both of them have been decreased. It had been shown that geranyl acetate might be biotransformed to geraniol and then geraniol is biotransformed to nerol.

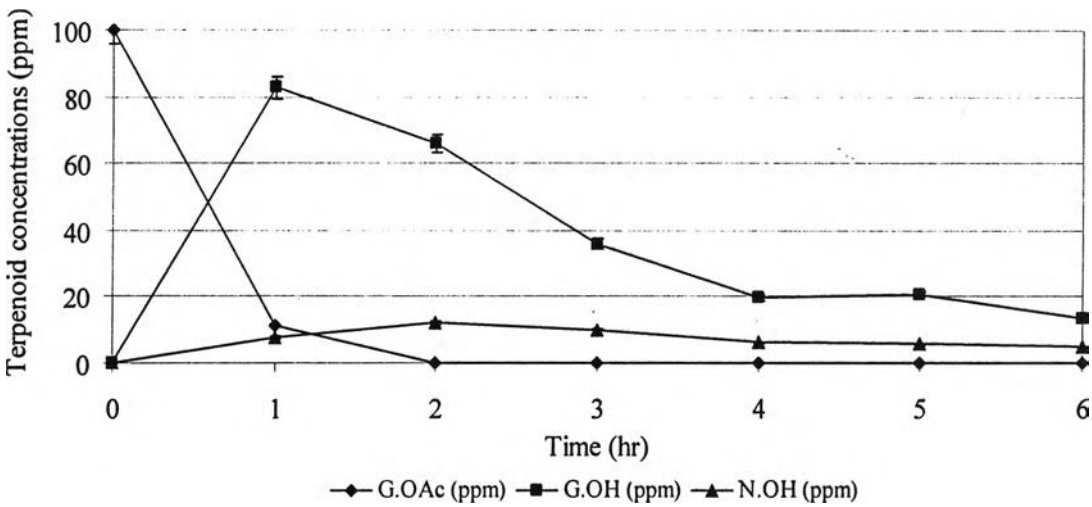
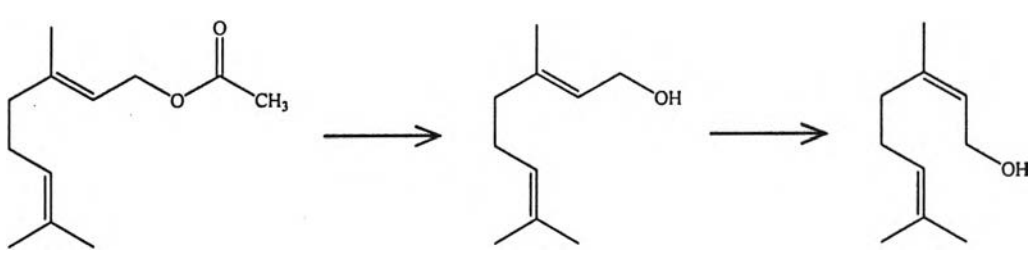



Figure 46 Terpenoid concentrations after feeding 100 ppm geranyl acetate in *Fortunella japonica* cell suspension cultures

The possible pathway of biotransformation of geranyl acetate in *Fortunella japonica* is shown in Fig. 47 below.




geranyl acetate geraniol nerol

Figure 47 The possible pathway of biotransformation of geranyl acetate in *Fortunella japonica*

#### 4.11.1.4 Substrate feeding in cell suspension cultures

##### 4.11.1.4.1 Biotransformation of acyclic terpenes

Biotransformation of acyclic monoterpenes and sesquiterpenes in cell suspension cultures of *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica*, and *Pogostemon cablin* were investigated by feeding citral, citronellal, citronellol, farnesol, farnesyl acetate, geraniol, geranyl acetate, linalool, linalyl acetate, myrcene, and nerol. Biotransformation products are shown in Table 44 and Fig. 48-57.

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Citral | *A. vulgaris* var. *indica* | Geraniol | 10.4 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Nerol | 8.3 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | *C. cyminum* | Geraniol | 12.8 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Nerol | 9.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | *F. japonica* | Geraniol | 14.9 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Nerol | 10.2 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | *P. cablin* | Geraniol | 10.8 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Nerol | 8.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |

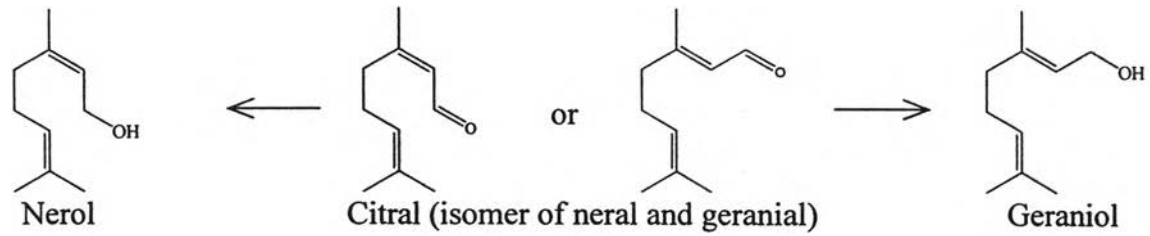



Figure 48 Biotransformation products of citral in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Citronellal | *A. vulgaris* var. *indica* | Citronellol | 6.4 | 156 | 1228 | 41, 55, 69, 81, 95, 109, 123, 138 |
| | *C. cyminum* | Citronellic acid | 18.3 | 184 | Data not shown | Data not shown |
| | | Citronellyl acetate | 14.5 | 198 | 1354 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | *F. japonica* | Citronellic acid | 20.5 | 184 | Data not shown | Data not shown |
| | | Citronellyl acetate | 15.5 | 198 | 1354 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | | Citronellol | 8.5 | 156 | 1228 | 41, 55, 69, 81, 95, 109, 123, 138 |
| | *P. cablin* | Citronellol | 7.5 | 156 | 1228 | 41, 55, 69, 81, 95, 109, 123, 138 |

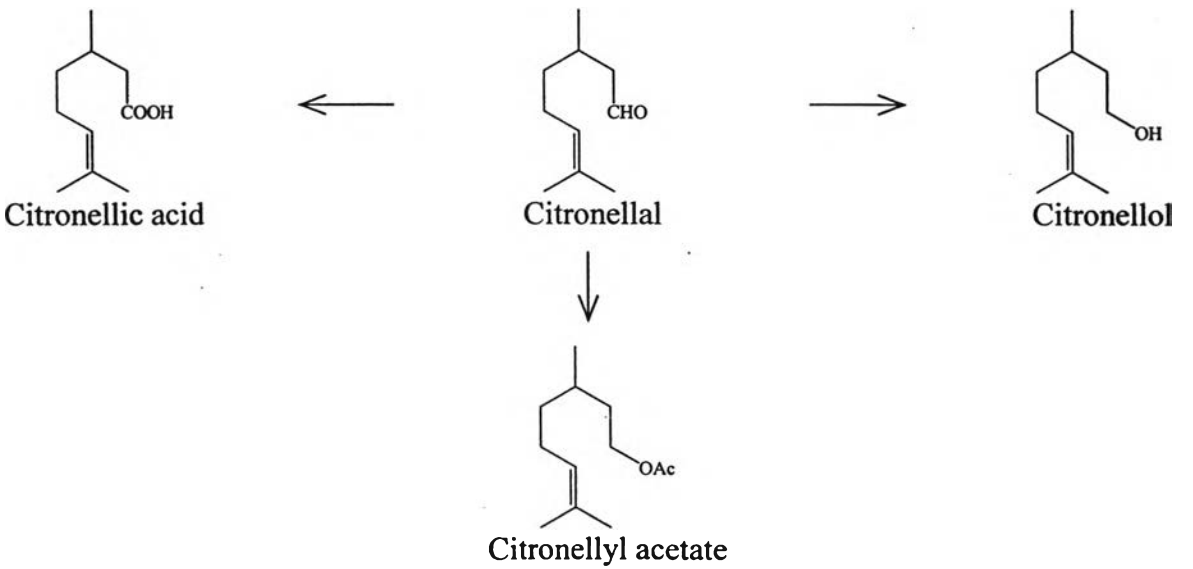



Figure 49 Biotransformation products of citronellal in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Citronellol | *A. vulgaris* var. *indica* | Citronellic acid | 8.6 | 184 | Data not shown | Data not shown |
| | *C. cyminum* | Terpinolene | 7.9 | 136 | 1088 | 41, 51, 67, 77, 79, 91, 93, 105, 121, 136 |
| | | α-Terpineol | 9.1 | 154 | 1189 | 41, 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Citronellic acid | 11.2 | 184 | Data not shown | Data not shown |
| | | Citronellyl acetate | 11.4 | 198 | 1354 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | | β-Elemene | 7.5 | 204 | 1391 | 41, 53, 67, 79, 93, 105, 121, 133, 147, 161, 175, 189 |
| | *F. japonica* | cis-p-Menth-2-en-1-ol | 15.5 | 154 | 1121 | 43, 55, 69, 81, 93, 111, 121, 139, 154 |
| | | Citronellal | 19.5 | 154 | 1153 | 41, 55, 69, 84, 95, 111, 121, 139, 154 |
| | | α-Terpineol | 14.3 | 154 | 1189 | 41, 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Citronellyl acetate | 15.8 | 198 | 1354 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | *P. cablin* | Citronellic acid | 9.5 | 184 | Data not shown | Data not shown |

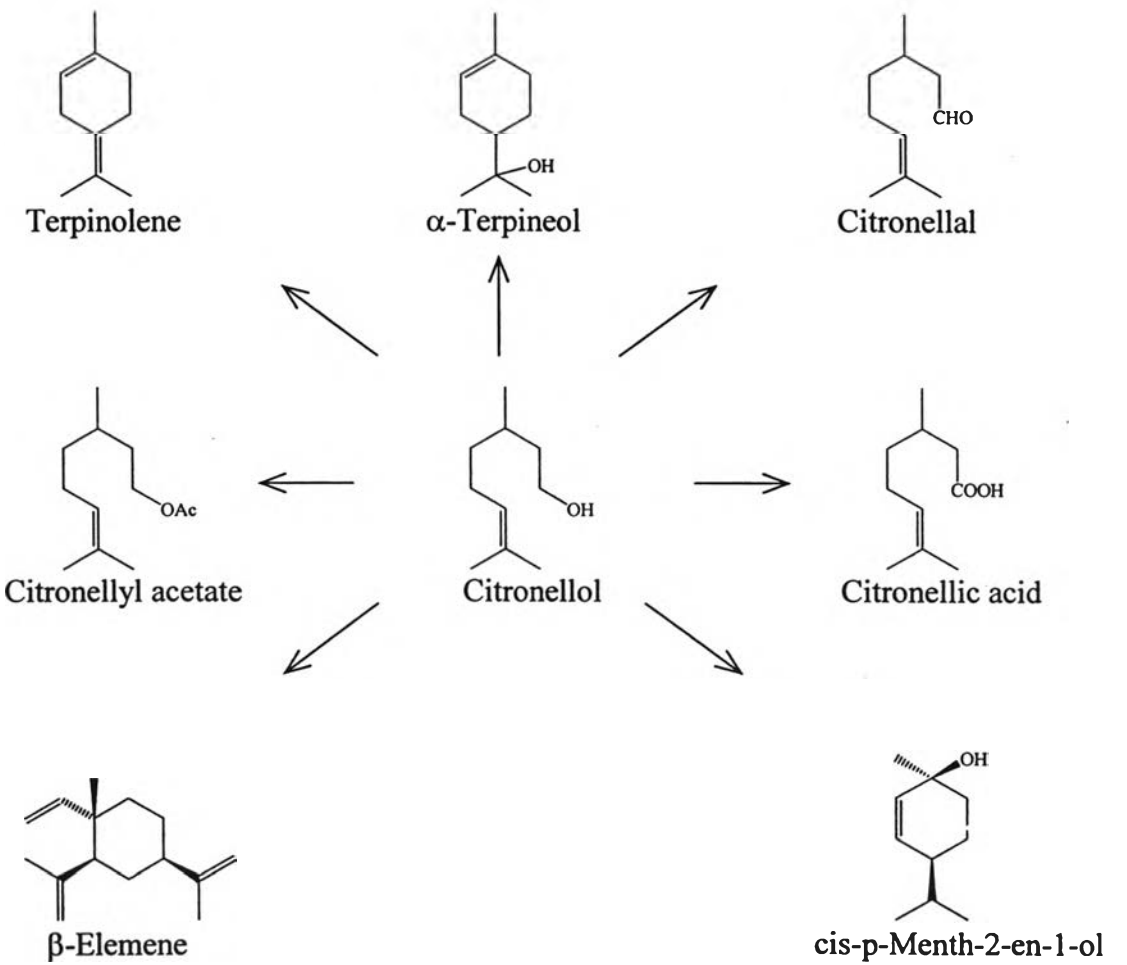
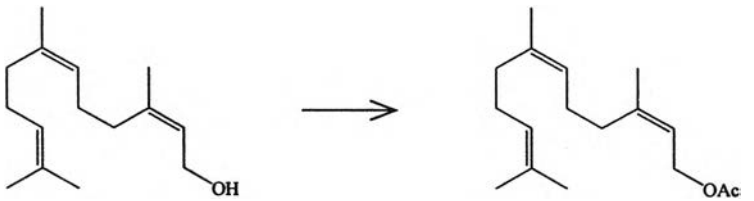



Figure 50 Biotransformation products of citronellol in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| *Z,Z*-Farnesol | *A. vulgaris* var. *indica* | *Z,Z*-Farnesyl acetate | 7.5 | 264 | 1843 | 41, 55, 69, 81, 93, 107, 121, 136, 161, 189 |
| | *C. cyminum* | *Z,Z*-Farnesyl acetate | 15.2 | 264 | 1843 | 41, 55, 69, 81, 93, 107, 121, 136, 161, 189 |
| | *F. japonica* | *Z,Z*-Farnesyl acetate | 19.3 | 264 | 1843 | 41, 55, 69, 81, 93, 107, 121, 136, 161, 189 |
| | *P. cablin* | *Z,Z*-Farnesyl acetate | 9.1 | 264 | 1843 | 41, 55, 69, 81, 93, 107, 121, 136, 161, 189 |

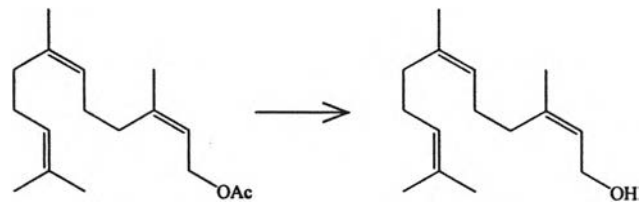



*Z,Z*-Farnesol *Z,Z*-Farnesyl acetate

Figure 51 Biotransformation products of *Z,Z*-farnesol in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| *Z,Z*-Farnesyl acetate | *A. vulgaris* var. *indica* | *Z,Z*-Farnesol | 15.5 | 222 | 1722 | 41, 53, 69, 81, 93, 107, 121, 137, 149, 161, 189 |
| | *C. cyminum* | *Z,Z*-Farnesol | 19.3 | 222 | 1722 | 41, 53, 69, 81, 93, 107, 121, 137, 149, 161, 189 |
| | *F. japonica* | *Z,Z*-Farnesol | 26.5 | 222 | 1722 | 41, 53, 69, 81, 93, 107, 121, 137, 149, 161, 189 |
| | *P. cablin* | *Z,Z*-Farnesol | 18.5 | 222 | 1722 | 41, 53, 69, 81, 93, 107, 121, 137, 149, 161, 189 |




*Z,Z*-Farnesyl acetate *Z,Z*-Farnesol

Figure 52 Biotransformation products of *Z,Z*-farnesyl acetate in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Geraniol | *A. vulgaris* var. *indica* | Nerol | 19.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | | Linalool | 18.5 | 154 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| | *C. cyminum* | Nerol | 20.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | *F. japonica* | α-Terpineol | 4.2 | 154 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Linalyl acetate | 7.3 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |
| | | Geranial | 12.5 | 152 | 1270 | 41, 53, 59, 69, 83, 95, 109, 123, 137, 152 |
| | *P. cablin* | Nerol | 15.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | | Geranial | 9.5 | 152 | 1270 | 41, 53, 59, 69, 83, 95, 109, 123, 137, 152 |

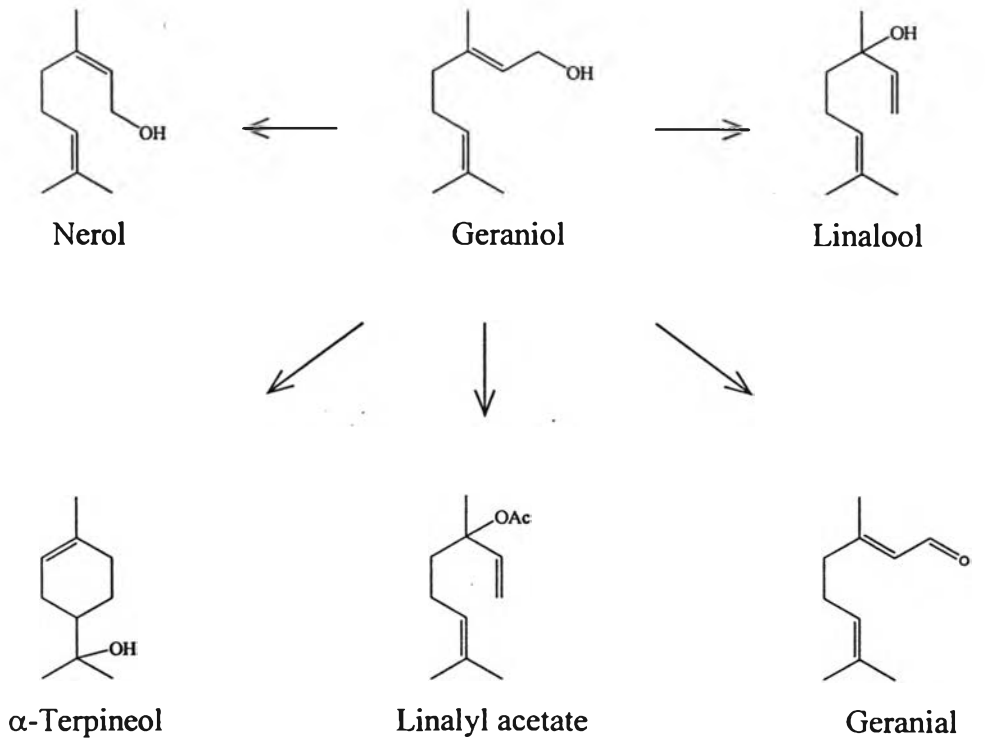



Figure 53 Biotransformation products of geraniol in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Geranyl acetate | *A. vulgaris* var. *indica* | Geraniol | 15.2 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Geranial | 9.5 | 152 | 1270 | 41, 53, 59, 69, 83, 95, 109, 123, 137, 152 |
| | *C. cyminum* | Nerol | 11.5 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | | Geraniol | 14.5 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | *F. japonica* | α-Terpinene | 4.7 | 136 | 1018 | 41, 55, 60, 65, 77, 93, 105, 121, 136 |
| | | Nerol | 12.2 | 154 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| | | Geraniol | 15.4 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | *P. cablin* | Geraniol | 16.8 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

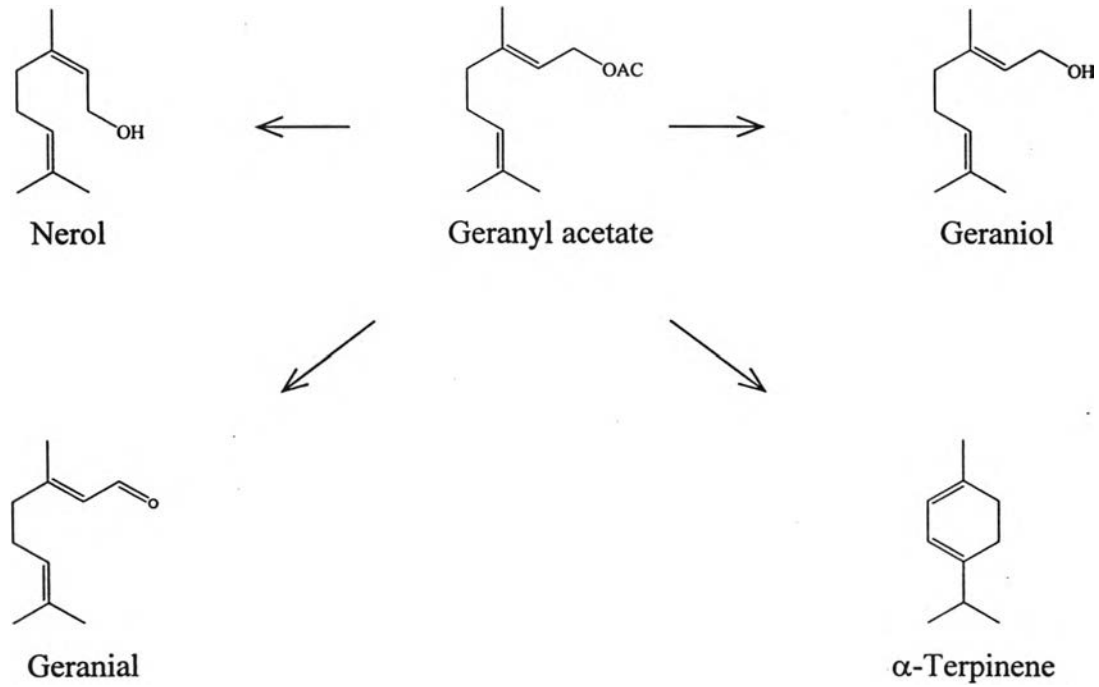



Figure 54 Biotransformation products of geranyl acetate in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Linalool | *A. vulgaris* var. *indica* | Linayl acetate | 12.5 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |
| | *C. cyminum* | Linalyl acetate | 16.5 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |
| | *F. japonica* | cis-Linalool oxide | 15.4 | 170 | 1074 | 43, 59, 67, 79, 93, 111, 125, 137, 153, 164 |
| | | α-Terpineol | 14.4 | 154 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Linalyl acetate | 17.6 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |
| | | Menthyl acetate | 17.6 | 198 | 1294 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | | Citronellic acid | 15.63 | 184 | Data not shown | Data not shown |
| | | Geranyl acetate | 14.3 | 198 | 1383 | 41, 43, 53, 69, 80, 93, 107, 121, 136 |
| | *P. cablin* | Linalyl acetate | 11.5 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |

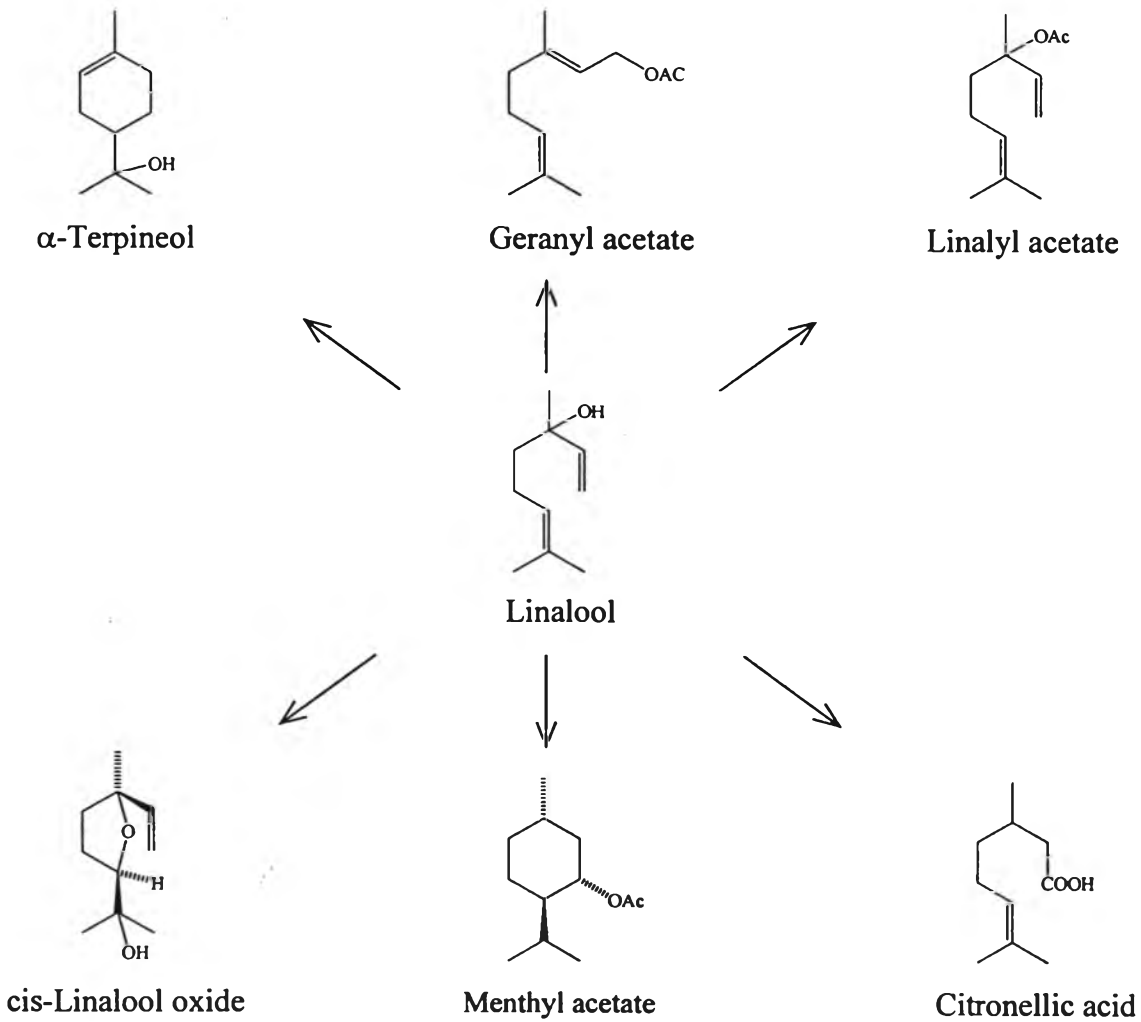



Figure 55 Biotransformation products of linalool in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Linalyl acetate | *A. vulgaris* var. *indica* | Linalool | 13.5 | 154 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| | *C. cyminum* | Linalool | 17.3 | 154 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| | | α-Terpineol | 11.6 | 154 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Geraniol | 5.9 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | α-Terpinyl acetate | 4.5 | 196 | 1350 | 43, 55, 67, 79, 93, 105, 121, 136 |
| | | Neryl acetate | 4.5 | 196 | 1365 | 41, 43, 69, 80, 93, 107, 121, 136, 154 |

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Linalyl acetate | *F. japonica* | β-Myrcene | 1.17 | 136 | 991 | 41, 53, 69, 81, 93, 107, 121, 136 |
| | | d-Limonene | 0.8 | 136 | 1031 | 41, 53, 67, 79, 93, 107, 121, 136 |
| | | cis-Linalool oxide | 1.58 | 170 | 1074 | 43, 59, 67, 79, 93, 111, 125, 137, 153, 164 |
| | | Linalool | 18.2 | 154 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| | | Terpinen-4-ol | 4.2 | 154 | 1177 | 43, 55, 67, 71, 81, 93, 98, 111, 125, 136, 154 |
| | | α-Terpineol | 12.6 | 154 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | α-Terpinyl acetate | 9.5 | 196 | 1350 | 43, 55, 67, 79, 93, 105, 121, 136 |
| | | Geranyl acetate | 5.7 | 196 | 1383 | 41, 53, 69, 80, 93, 107, 121, 136 |
| | | trans-Caryophyllene | 4.7 | 204 | 1418 | 41, 55, 69, 79, 91, 105, 119, 133, 147, 161, 175, 189, 204 |
| | *P. cablin* | Linalool | 16.5 | 154 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |

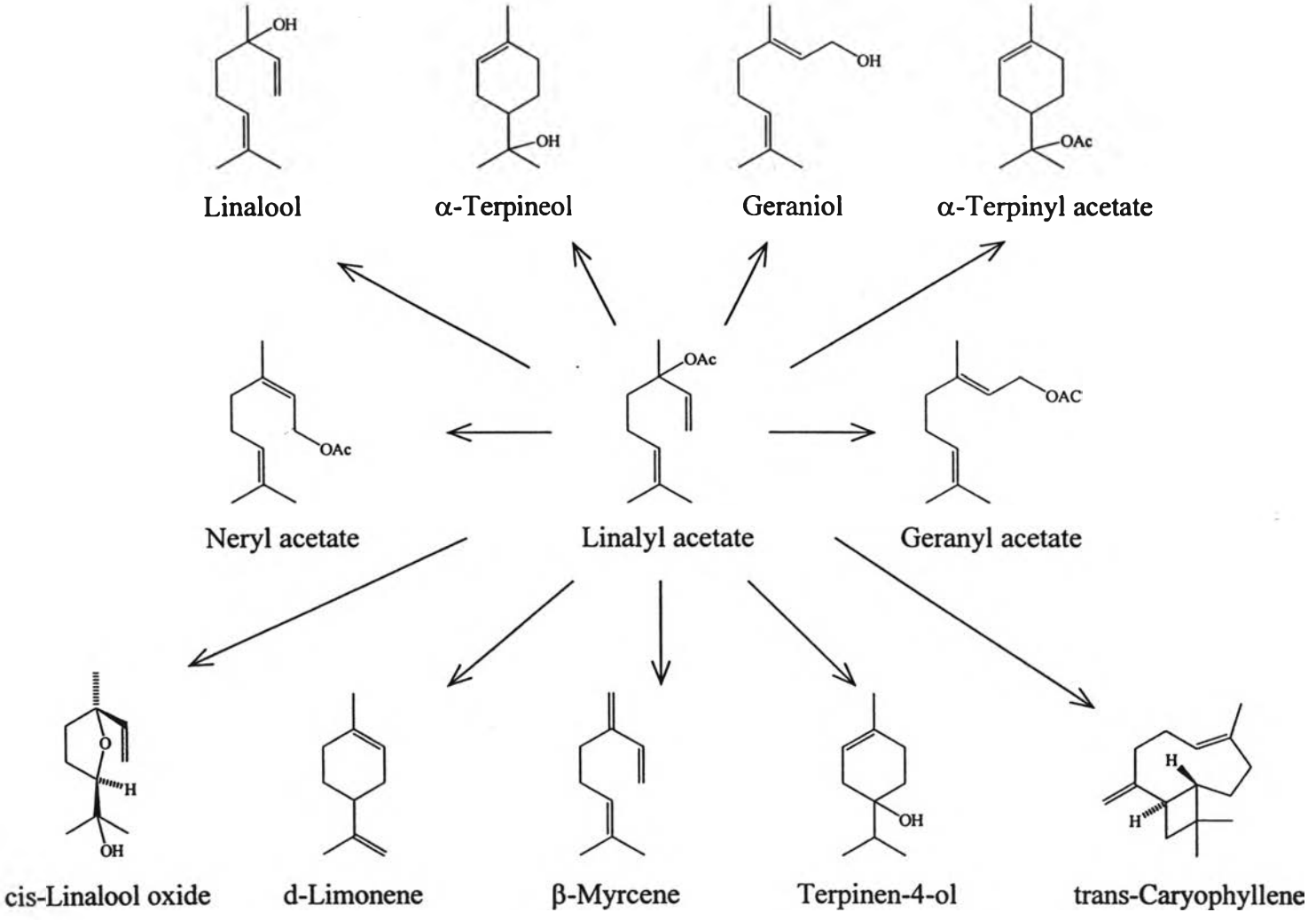



Figure 56 Biotransformation products of linalyl acetate in individual cell suspension cultures

Table 44 Biotransformation products from a range of acyclic terpenes fed to individual suspensions (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Nerol | *A. vulgaris* var. *indica* | Geraniol | 16.5 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | *C. cyminum* | Geraniol | 18.5 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |
| | | Neral | 14.5 | 152 | 1240 | 41, 53, 59, 69, 81, 95, 99, 109, 119, 137 |
| | | Neryl acetate | 9.5 | 196 | 1365 | 41, 53, 69, 80, 93, 107, 121, 136, 154 |
| | *F. japonica* | α-Terpineol | 14.7 | 154 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| | | Linalyl acetate | 16.7 | 196 | 1257 | 43, 55, 67, 80, 93, 105, 121, 136 |
| | | Geranial | 15.5 | 152 | 1270 | 41, 53, 59, 69, 83, 95, 109, 123, 137, 152 |
| | | trans-Dihydro-α-terpineol | 16.1 | 156 | 1161 | 49, 59, 67, 81, 95, 123, 155 |
| | *P. cablin* | Geraniol | 13.5 | 154 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

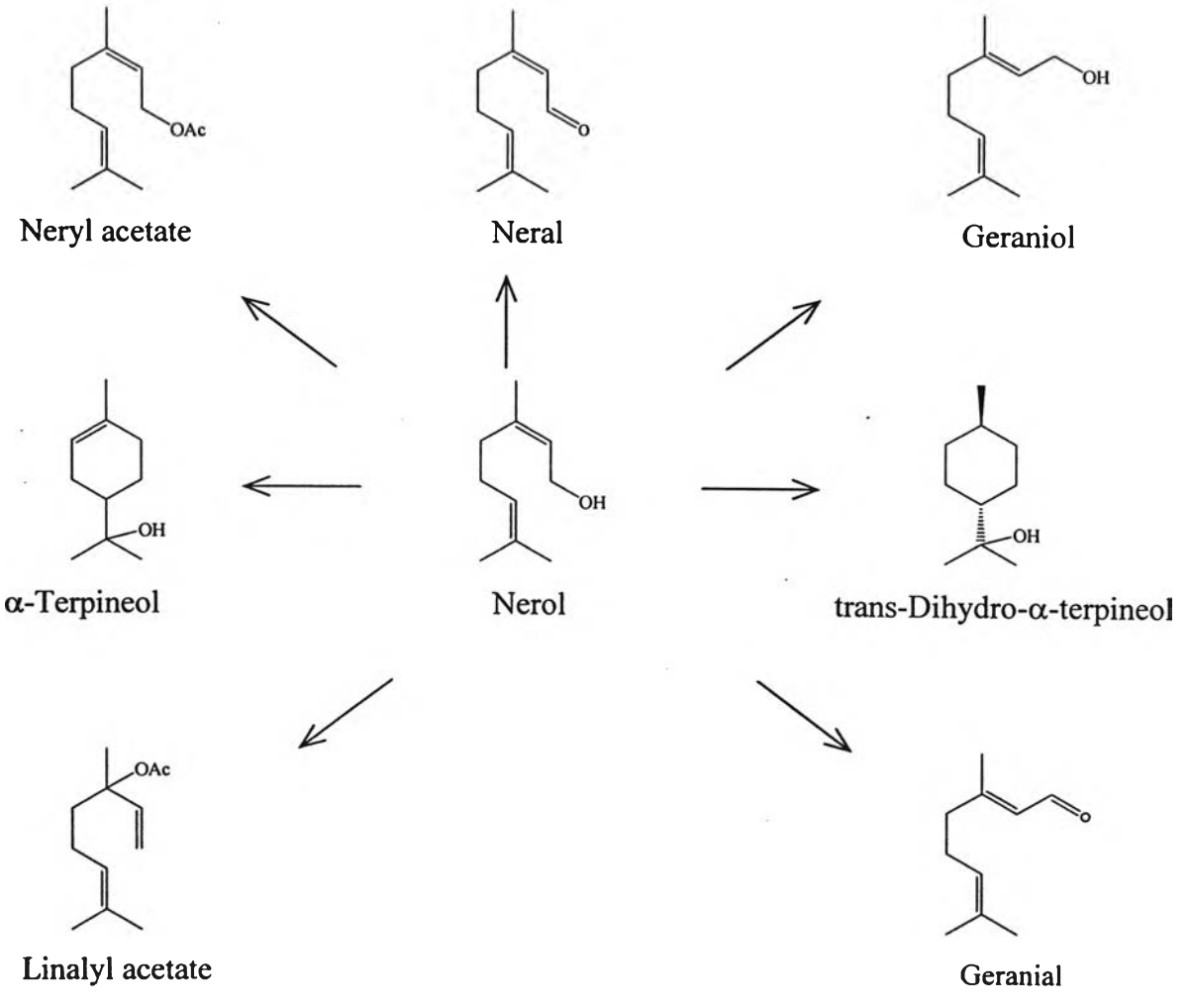



Figure 57 Biotransformation products of nerol in individual cell suspension cultures

#### 4.11.1.4.2 Biotransformation of cyclic monoterpenes

Some common cyclic monoterpenes were administered to suspension cultures of *Artemisia vulgaris* var. *indica*, *Cuminum cyminum*, *Fortunella japonica*, and *Pogostemon cablin*. Again the results showed different biotransformation products in individual suspensions. Details are shown in Table 45 and Fig. 58-67.

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| l-Bornyl acetate | *A. vulgaris* var. *indica* | l-Borneol | 20.1 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |
| | *C. cyminum* | Camphor | 11.2 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |
| | | l-Borneol | 21.7 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |
| | *F. japonica* | Camphor | 14.8 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |
| | | l-Borneol | 25.6 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |
| | *P. cablin* | l-Borneol | 20.3 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |

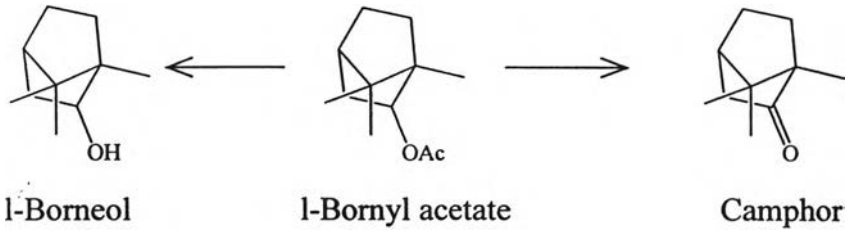
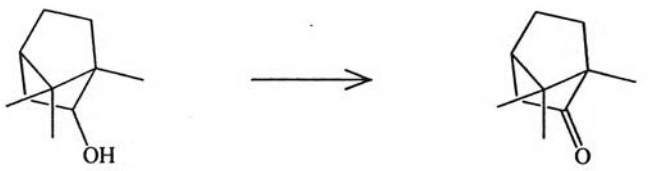



Figure 58 Biotransformation products of l-bornyl acetate in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| l-Borneol | *A. vulgaris* var. *indica* | Camphor | 20.2 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |
| | *C. cyminum* | Camphor | 25.2 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |
| | *F. japonica* | Camphor | 28.5 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |
| | *P. cablin* | Camphor | 21.9 | 152 | 1143 | 41, 55, 67, 81, 95, 108, 137, 152 |

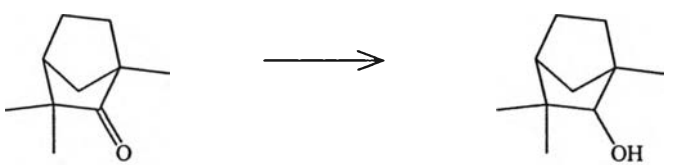



l-Borneol Camphor

Figure 59 Biotransformation products of l-borneol in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Fenchone | *A. vulgaris* var. *indica* | Fenchol | 21.3 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | *C. cyminum* | Fenchol | 24.6 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | *F. japonica* | Fenchol | 28.2 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | *P. cablin* | Fenchol | 22.8 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |

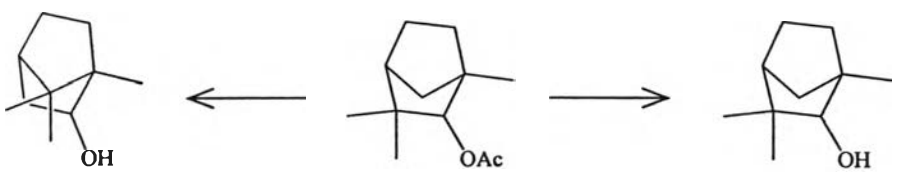



Fenchone Fenchol

Figure 60 Biotransformation products of fenchone in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Fenchyl acetate | *A. vulgaris* var. *indica* | Fenchol | 14.3 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | *C. cyminum* | Fenchol | 18.3 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | | Borneol | 9.2 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |
| | *F. japonica* | Fenchol | 23.5 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |
| | | Borneol | 12.1 | 154 | 1165 | 41, 55, 67, 81, 95, 111, 121, 137 |
| | *P. cablin* | Fenchol | 14.6 | 154 | 1112 | 43, 57, 67, 81, 93, 98, 111, 121, 137 |

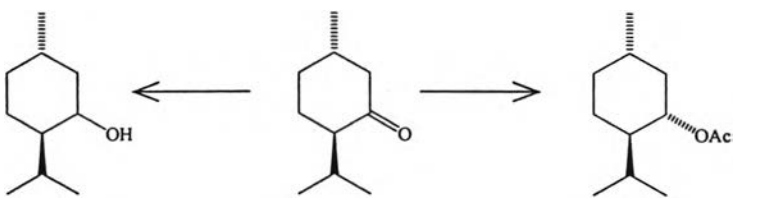



Borneol Fenchyl acetate Fenchol

Figure 61 Biotransformation products of fenchyl acetate in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Menthone | *A. vulgaris* var. *indica* | Menthol | 5.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | *C. cyminum* | Menthol | 9.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | *F. japonica* | Menthol | 12.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | | Menthyl acetate | 7.5 | 198 | 1294 | 43, 55, 67, 81, 95, 109, 123, 138 |
| | *P. cablin* | Menthol | 6.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |

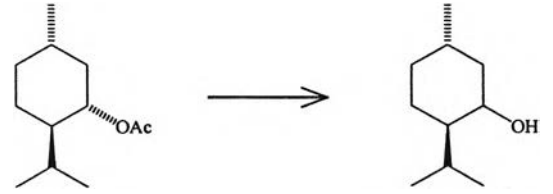



Menthol Menthone Menthyl acetate

Figure 62 Biotransformation products of menthone in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Menthyl acetate | *A. vulgaris* var. *indica* | Menthol | 5.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | *C. cyminum* | Menthol | 6.7 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | *F. japonica* | Menthol | 9.5 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |
| | *P. cablin* | Menthol | 5.3 | 156 | 1173 | 41, 55, 71, 81, 95, 109, 123, 138 |




Menthyl acetate Menthol

Figure 63 Biotransformation products of menthyl acetate in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| d-Limonene | *A. vulgaris* var. *indica* | α-Terpineol | 8.5 | 154 | 1189 | 43, 59, 67, 81, 93, 107, 121, 136 |
| | | cis-Carveol | 4.2 | 152 | 1229 | 41, 55, 67, 84, 93, 109, 119, 134 |
| | *C. cyminum* | α-Terpineol | 11.5 | 154 | 1189 | 43, 59, 67, 81, 93, 107, 121, 136 |
| | | cis-Carveol | 4.5 | 152 | 1229 | 41, 55, 67, 84, 93, 109, 119, 134 |
| | *F. japonica* | α-Terpineol | 13.5 | 154 | 1189 | 43, 59, 67, 81, 93, 107, 121, 136 |
| | | cis-Carveol | 5.8 | 152 | 1229 | 41, 55, 67, 84, 93, 109, 119, 134 |
| | *P. cablin* | α-Terpineol | 8.3 | 154 | 1189 | 43, 59, 67, 81, 93, 107, 121, 136 |
| | | cis-Carveol | 3.9 | 152 | 1229 | 41, 55, 67, 84, 93, 109, 119, 134 |

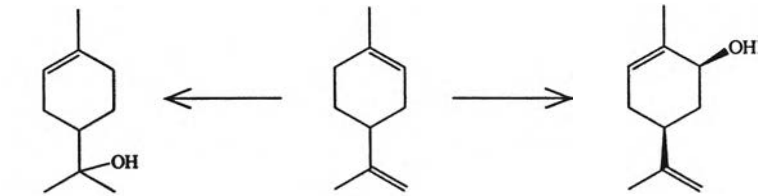
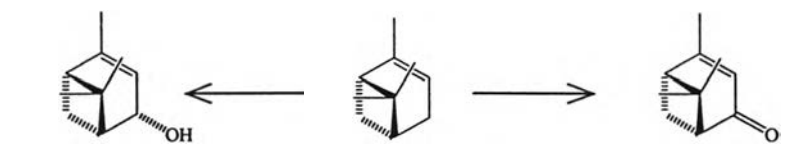



Figure 64 Biotransformation products of d-limonene in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| α-Pinene | *A. vulgaris* var. *indica* | trans-Verbenol | 11.5 | 152 | 1144 | 41, 55, 67, 81, 91, 109, 119 |
| | *C. cyminum* | trans-Verbenol | 16.3 | 152 | 1144 | 41, 55, 67, 81, 91, 109, 119 |
| | | Verbenone | 6.5 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |
| | *F. japonica* | trans-Verbenol | 18.5 | 152 | 1144 | 41, 55, 67, 81, 91, 109, 119 |
| | | Verbenone | 7.2 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |
| | *P. cablin* | trans-Verbenol | 11.2 | 152 | 1144 | 41, 55, 67, 81, 91, 109, 119 |




trans-Verbenol α-Pinene Verbenone

Figure 65 Biotransformation products of α-pinene in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| β-Pinene | *A. vulgaris* var. *indica* | Myrtenol | 16.8 | 152 | 1194 | 41, 53, 67, 79, 91, 107, 119 137 152 |
| | *C. cyminum* | Myrtenol | 21.5 | 152 | 1194 | 41, 53, 67, 79, 91, 107, 119 137 152 |
| | | Myrtenal | 25.5 | 150 | 1193 | 41, 51, 67, 79, 91, 107, 121, 135, 150 |
| | *F. japonica* | Myrtenol | 24.8 | 152 | 1194 | 41, 53, 67, 79, 91, 107, 119 137 152 |
| | | Myrtenal | 28.3 | 150 | 1193 | 41, 51, 67, 79, 91, 107, 121, 135, 150 |
| | *P. cablin* | Myrtenol | 18.6 | 152 | 1194 | 41, 53, 67, 79, 91, 107, 119 137 152 |

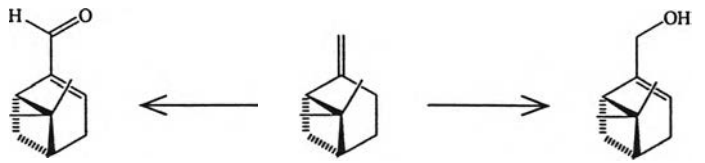
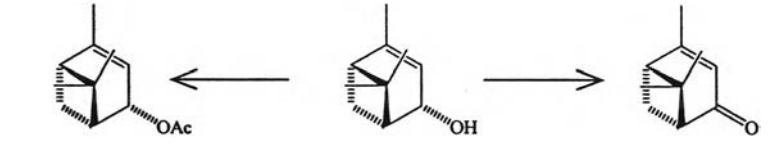



Figure 66 Biotransformation products of β-pinene in individual cell suspension cultures

Table 45 Biotransformation products from a range of cyclic terpenes fed to individual cell suspension cultures (Reading taken at 24 hours unless otherwise stated) (Cont.)

| Substrate | Species | Biotransformation products | Yield (%) | MW | Kovat's index | MS data |
|---|---|---|---|---|---|---|
| Verbenol | *A. vulgaris* var. *indica* | Verbenone | 22.4 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |
| | *C. cyminum* | Verbenone | 24.3 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |
| | | Verbenyl acetate | 21.9 | 194 | 1282 | 43, 59, 67, 77, 91, 109, 119, 134 |
| | *F. japonica* | Verbenone | 29.5 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |
| | | Verbenyl acetate | 23.5 | 194 | 1282 | 43, 59, 67, 77, 91, 109, 119, 134 |
| | *P. cablin* | Verbenone | 10.5 | 150 | 1204 | 41, 55, 67, 79, 91, 107, 122, 135, 150 |




Verbenyl acetate Verbenol Verbenone

Figure 67 Biotransformation products of verbenol in individual cell suspension cultures

#### 4.11.1.5 Improved yield of biotransformation products by p-HEMA discs

Various ratio of HEMA, EGDMA and water content were chosen in order to optimise the ratio of disc components. The suitable ratio of HEMA, EGDMA and water content studied by Zhu (Zhu and Lockwood, 2000) was 5:0.5:2. This ratio had been selected to prepare polymer disc in this experiment. 100 ppm Geraniol was added into p-HEMA disc and its time-course studied after feeding to cell cultures.

##### 4.11.1.5.1 Time-course study of feeding of p-HEMA discs containing monoterpenes to cell suspension cultures

According to experiment 4.11.1.3, after feeding 100 ppm geraniol into cell suspension cultures of *Fortunella japonica*, it had been biotransformed to nerol and geranyl acetate. However, the levels of geraniol, nerol, and geranyl acetate were decreased rapidly, meanwhile neryl acetate could not be detected at any times. In order to improved yields of biotransformation products, the disc polymer named p-HEMA disc was applied.

After feeding p-HEMA disc containing 100 ppm geraniol, geraniol was control-released from disc polymer and biotransformed to nerol and geranyl acetate. Nerol and geranyl acetate could be detected until 21 day at the low level and stable concentrations in cell cultures. Moreover, neryl acetate could be detected at the lowest concentration.

#### 4.11.1.6 Feeding precursors of each major chemical constituents in individual cell suspension cultures

In attempt to increase the level of major chemical constituents, the precursor of each compound was fed into cell suspension cultures in the early of stationary phase. After feeding precursor experiments of individual cell cultures, their levels of major chemical constituents were successfully increased as shown in Table 46.

Table 46 Yield of major chemical constituent of individual plant after precursor feeding experiments

| **Plant species** | **Precursor feeding** | **Major chemical constituent** | **Yield (ppm) Control experiment** | **Yield (ppm) Feeding experiment** |
|---|---|---|---|---|
| *Artemisia vulgaris* var. *indica* | Geraniol | (+)-davanone | 21.5 | 35.5 |
| *Fortunella japonica* | Geraniol | d-limonene | 22.5 | 26.5 |
| | Nerol | d-limonene | 22.5 | 28.9 |
| | Linalool | d-limonene | 22.5 | 40.4 |
| *Cuminum cyminum* | Gerniol | cuminaldehyde | 26.7 | 31.5 |
| | Cuminol | cuminaldehyde | 26.7 | 52.5 |
| *Pogostemon cablin* | Z,Z-Farnesol | patchouli alcohol | 19.5 | 25.5 |

### 4.11.2 Elicitation

In attempt to increase d-limonene level in cell suspension cultures of *Fortunella japonica*, chitosan and methyl jasmonate had been used as elicitors in elicitation.

#### 4.11.2.1 Elicitation with chitosan

Chitosan has been proved to be the effective elicitor used for increasing the level of secondary metabolites in cell cultures. According to it is permeabilising agent as well, excessively chitosan concentration may be caused cell death. Determination of the optimum chitosan concentration should be done before starting elicitation experiment.

##### 4.11.2.1.1 Determination of optimum chitosan concentration

To determine the optimum chitosan concentration, the concentrations of chitosan were varied from 50 to 400 ppm, and the effect of chitosan on cell growth and volatile constituents, particularly in d-limonene, was investigated for 7 days. As shown in Fig. 68, any concentrations of chitosan up to 200 ppm did not inhibit growth of *Fortunella japonica* and d-limonene was reached a maximum value at 200 ppm chitosan.

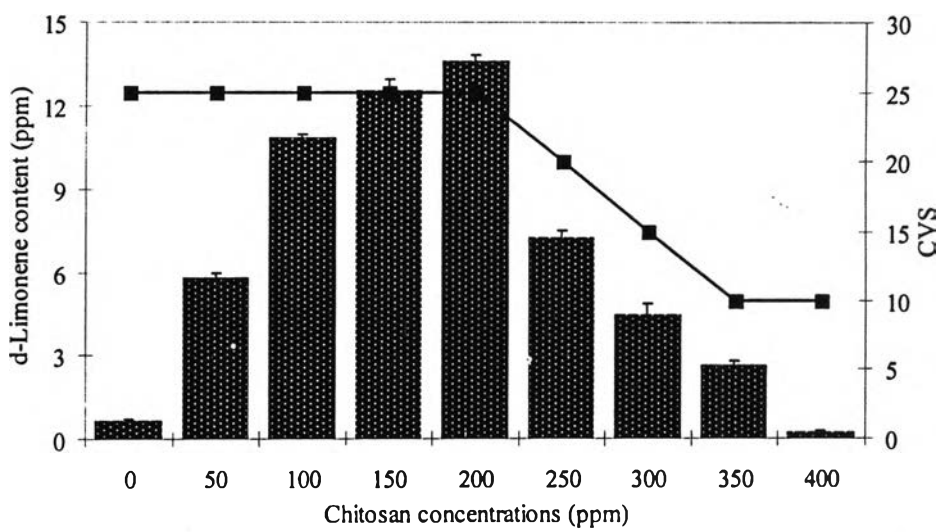



Figure 68 The effect of various chitosan concentrations on cell growth ( ) and d-limonene production ( ) in *F. japonica* suspension cultures after 7 days

#### 4.11.2.1.2 Determination of optimum period of elicitation

200 ppm of chitosan was added to suspension cultures of *Fortunella japonica*, and volatile constituents, particularly in d-limonene, were investigated for 21 days. The result has been shown in Fig. 69. In elicited cell, d-limonene content increased up at day 12 and then decrease, whilst d-limonene content in control experiment remained low until day 21. The maximum d-limonene content reached 42.5 ppm (34.27 fold increase compared to control) at day 12 of elicitation. This result indicates that the optimum period of elicitation for d-limonene production is 12 days. From the pathways for α-terpineol formation, it is formed from d-limonene or linalool. In this experiment, after d-limonene decrease in day 15, α-terpineol increase. Both d-limonene and α-terpineol remain in culture until day 21, 17 fold and 15 fold increase, respectively, compare to control.

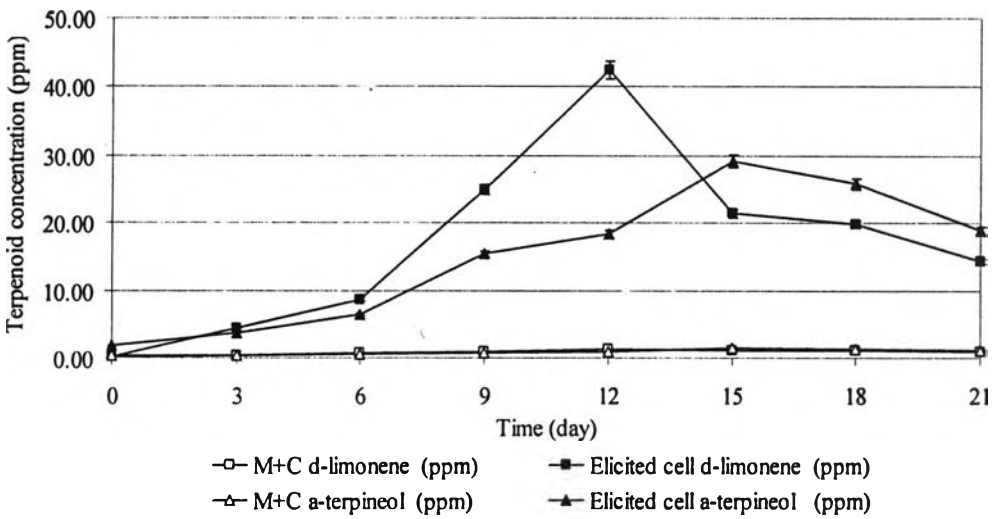



Fig. 69 d-Limonene and α-tepineol concentrations in elicited cell cultures of *Fortunella japonica* compared to control experiment (non-elicited cells; M+C)

#### 4.11.2.2 Elicitation with methyl jasmonate (MEJA)

##### 4.11.2.2.1 Determination of optimum concentration of methyl jasmonate

The concentration of MEJA was varied from 50 to 200 ppm. The effect of MEJA on cell growth and volatile constituents are investigated every 7 day until 21 days. After 21 days of experiment, 50 ppm MEJA seemed to inhibit growth of *Fortunella japonica* cell suspension cultures, whilst cell did not growth in 100 and 200 ppm MEJA. Meanwhile, every concentrations of MEJA effect to cell growth of *Cuminum cyminum*, their growth rate were less than control experiment. Moreover, the pH of both cultures compared to control experiment was different.

##### 4.11.2.2.2 Effect of methyl jasmonate on essential oil constituents product in *Fortunella japonica* cell suspension cultures

By day 14 of the experiment in *Fortunella japonica* cell suspension cultures, the GC chromatogram shows peaks of nerol (4.06 ppm) and geraniol (5.41 ppm), while linalool shows only a trace. By day 21 of the experiment, all of them had disappeared, but the GC chromatogram showed peaks of α-thujene (5.83 ppm), camphene (7.17 ppm), β-pinene (6.17 ppm), isopulegol (15.21 ppm) and geranyl acetate (17.07 ppm). α-Thujene, camphene, and β-pinene are volatile constituents found in leaves essential oil, but isopulegol and geranyl acetate are not. The results have been shown in Table 47 and Table 48, respectively.

Table 47 Detected essential oil constituents after day 14 of elicitation of methyl jasmonate in *Fortunella japonica*

| Detected compound | Yield (ppm) | KI | MS data |
|---|---|---|---|
| linalool | t | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| nerol | 4.07 | 1228 | 41, 53, 69, 81, 93, 111, 121, 139 |
| geraniol | 5.41 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

t = trace (less than 0.01)

Table 48 Detected essential oil constituents after day 21 of elicitation of methyl jasmonate in *Fortunella japonica*

| **Detected compound** | **Yield (ppm)** | **KI** | **MS data** |
|---|---|---|---|
| α-thujene | 5.83 | 931 | 41, 51, 65, 77, 91, 105, 121, 136 |
| camphene | 7.17 | 953 | 41, 53, 67, 79, 93, 107, 121, 136 |
| β-pinene | 6.17 | 980 | 41, 53, 69, 79, 93, 107, 121, 136 |
| isopulegol | 15.21 | 1146 | 41, 55, 67, 81, 95, 111, 121, 137 |
| geranyl acetate | 17.07 | 1383 | 41, 53, 69, 80, 93, 107, 121, 136 |

### 4.11.3 Permeabilisation

In an attempt to increase levels of essential oil constituents, particularly in d-limonene, Tween-20 was used as permeabilising agent in this experiment.

#### 4.11.3.1 Determination of optimum Tween-20 concentration

Tween-20 is permeabilising agent, and excessive Tween-20 concentration may cause cell death. The optimum Tween-20 concentration should be determine before starting permeabilisation experiment.

The concentration of Tween-20 was varied (1, 1.5, and 2 % (w/v), and effect of Tween-20 concentration on appearance, growth rate, and volatile constituents were investigated every 7 day, until 21 days.

At day 21 of the experiment no concentration of Tween-20 inhibited growth of *Fortunella japonica* cell suspension cultures. Meanwhile, every concentration of Tween-20 effected cell growth of *Cuminum cyminum*, their growth rate were less than control experiment.

The pH of both cultures compared to control experiment was different.

#### 4.11.3.2 Effect of Tween-20 on essential oil constituents in *Fortunella japonica* cell suspension cultures

In day 7 of experiment, there are nothing different between permeabilised cell and control experiment. In day 14 of experiment, the GC chromatogram of all Tween-20 concentrations showed peaks of linalool and geraniol, while nerol is only shown in GC chromatogram have 2% Tween-20. Their concentration in individual cell is shown below. Meanwhile, in day 21 of experiment, only GC chromatogram have 2% Tween-20 shows peaks of myrcenol, α-terpineol and geranyl acetate. The results have been shown in Table 49 and Table 50, respectively.

Table 49 Detected essential oil constituents after day 14 of permeabilisation using various Tween-20 concentrations in *Fortunella japonica*

**1% Tween-20**

| Detected compound | Yield (ppm) | KI | MS data |
|---|---|---|---|
| linalool | 4.63 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| geraniol | 6.53 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

**1.5 % Tween-20**

| Detected compound | Yield (ppm) | KI | MS data |
|---|---|---|---|
| linalool | 4.95 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| geraniol | 5.90 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

**2% Tween-20**

| Detected compound | Yield (ppm) | KI | MS data |
|---|---|---|---|
| linalool | 6.71 | 1098 | 43, 55, 71, 80, 93, 109, 121, 136, 154 |
| nerol | 4.41 | 1228 | 41, 53, 69, 81, 93, 111, 123, 139 |
| geraniol | 8.70 | 1255 | 41, 53, 69, 81, 93, 111, 123, 139 |

Table 50 Detected essential oil constituents after day 21of permeabilisation using 2% Tween-20 in *Fortunella japonica*

**2% Tween-20**

| Detected compound | Yield (ppm) | KI | MS data |
|---|---|---|---|
| myrcenol | 5.62 | 1118 | 43, 53, 59, 67, 79, 93, 107, 121, 136 |
| α-terpineol | 136.08 | 1189 | 43, 55, 59, 67, 71, 81, 93, 107, 121, 136 |
| geranyl acetate | 120.46 | 1383 | 41, 53, 69, 80, 93, 107, 121, 136 |

### 4.11.4 *In situ* product removal (two-phase system)

In order to prevent cell death from monoterpenes released in culture media, n-hexadecane was applied as the second phase in culture media for accumulating essential oil constituents. After this experiment, the levels of major chemical constituents of each plant cultures are not different significantly. They were still detected in low levels, however, each plant culture looked healthy compared to the control experiment.