# PRODUCTION OF INSECTICIDES AND ALKALOIDS USING PLANT TISSUE CULTURE METHODS

**Plant-Based Insecticides from Native and *In Vitro* Sources**

| Sl. No. | Insecticides | Plant | Family | Source tissue | Content | *In vitro* | Content |
|---|---|---|---|---|---|---|---|
| 1. | Pyrethrins | *Chrysanthemum cinerariaefolium* | Compositae | Flowers | 1–2% | Callus | 0.023–0.113% |
| | | | | | | Shoot cultures | 0.050–0.34% |
| | | *Tagetes erecta* | Compositae | Flowers | 0.9% | Suspension cultures | 1.16% |
| 2. | Azadirachtin | *Azadiracta indica* | Meliaceae | Seed Kernel | 0.14–1.66% | Microprop. Shoots | 2% |
| | | | | | — | Callus | 0.0007% |
| 3. | Nimbin | *Azadiracta indica* | Meliaceae | Bark | 0.4% | Cultures differentiating roots | 0.025% |
| 4. | Thiophenes | *Tagetes patula* | Compositae | Whole plant | 1% | Hairy root (α-terthienyl) | 0.0127 |
| | | | | | | Transformed callus | 0.0074% |
| 5. | Nicotine | *Nicotiana tabacum* | Solanaceae | Leaves | 2–5% | Callus | 2.14% |
| | | *Nicotiana rustica* | Solanaceae | Leaves | 5–14% | — | — |
| 6. | Rotenoids | *Derris elliptica* | Leguminosae | Roots | 5–9% | Callus with rootlets | 0.016% |
| | | | | | | Callus | 0.0003% |
| | | *Loncnocarpus utilis* | Leguminosae | Roots | 8–11% | — | — |
| | | *Crotoloria burhia* | Leguminosae | | | Callus | 1.35% |
| | | *Tephrosia purpurea* | Leguminosae | Leaves | 1–7% | Suspension cultures | 2.8% |
| | | *Tephrosia vogelli* | Leguminosae | — | — | Static cultures | 1.25% |
| 7. | Phytoecdysones | *Achyranthes* sp. | Amaranthaceae | — | — | Callus | 0.002% |
| | | *Triantnema portulacastrum* | Alzoceae | — | — | Callus | 0.036% |

The principal source of pyrethrins is *Chrysanthemum cinerariaefolium* (pyrethrum), where the achenes (ovules) of the flower heads contain approximately 94% of total pyrethrins (Brewer, 1973). The conventional production of pyrethrins is below global market requirements (Shand, 1992), and this inadequacy has necessitated production by *in vitro* methods.

ever, studies carried out on undifferentiated callus tissue gave varied responses for pyrethrin production. About 54% of 133 isolates cultured produced a 0.036% yield of pyrethrins (Zieg et al., 1983). A maximal yield of 0.224% was obtained at the end of the lag phase from high pyrethrin yielding a variety of pyrethrum (Ravishankar et al., 1989). It was observed that while a MS (Murashige and Skoog, 1962) medium with a carbon:nitrogen:phosphorus relative ratio of 2:1:2 was required for good leaf callus growth, hormonal supplementation with 2 mg $L^{-1}$ (2,4-dichlorophenoxy acetic acid) 2,4-D and 5 mg $L^{-1}$ kinetin gave maximal pyrethrin yield of 0.22% (Rajasekaran et al., 1990). While the presence of exogenous ascorbic acid was found to increase pyrethrin content in *in vitro* tissue of *Tagetes erecta* (Khanna and Khanna, 1976), supplementation of MS (Murashige and Skoog, 1962) with formaldehyde up to 0.02% enhanced pyrethrin production in *Chrysanthemum cinerariaefolium* (Nirmala et al., 1992), although it affected callus growth. Similarly, the presence of pyrethrins in callus was identified by GC and their activity tested by mosquito larvae bioassay (Aoki et

In the wake of the above studies, it was found that partially differentiated tissue culture is necessary for pyrethrin production, wherein the metabolite yields could be raised from barely detectable levels to approximately 0.5% dry weight. Analysis of shoots differentiated from callus revealed the presence of six pyrethrin esters and this was confirmed by GLC, GC-MS (Cashyap et al., 1978). Organized shoot cultures were shown to synthesize pyrethrins. Zieg et al. (1983) postulated that tissue organization may be necessary for the formation of specialized structures such as surface oil glands and internal secretory canals. The involvement of these was further confirmed by Head, 1966 and Zito et al., 1983. Although root organ cultures of *C. cinerariaefolium* showed an absence of pyrethrin production (Chumsri and Staba, 1975),

Bioconversions of readily available precursors in *Chrysanthemum* and *Tagetes* sp. by enzymatic synthesis from a cell-free homogenate containing plant enzymes and its incubation with a radioactively labeled mevalonic acid or isopentyl pyrophosphate was described in a patent granted to McLaughlin Gormley King Co., Minnesota, in 1984. The cloning of chrysanthemyl diphosphate synthase gene in a genetically engineered microorganism for developing a key active intermediate has been attempted by Agri-Dyne Technologies Inc., Utah (Shand, 1992), and the level of production and bioactivity testing are underway (King, 1993).

## AZADIRACHTIN PRODUCTION FROM NEEM EXPLANTS

Immature fruits of Neem plant were used as explants. The basal medium used in all experiments consisted of Murashige and Skoog (MS; 1962) macro- and microsalts, MS vitamins and 100 mg $L^{-1}$ myo-inositol. The pH of the medium was adjusted to 5.8 before autoclaving at 1.06 kg $cm^{-2}$ and 121 °C for 15 min. Cultures were maintained in 1000–2000 lux light intensity at 25 °C and cultured plant cells were harvested between 5 and 6 weeks and dried in an oven at 33 ± 2 °C until a constant weight was achieved. The drying temperature was kept low to prevent thermal decomposition of metabolites.

To establish cultures from zygotic embryo explants, immature fruits were collected and thoroughly washed in 1 % (v/v) savlon solution for 10 min, followed by rinsing with sterile distilled water (SDW). Fruits were then rinsed with 90 % ethanol for 30 s before surface sterilizing, using 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 10 min inside a laminar air-flow cabinet. After washing with SDW three times, the fruits were dissected with the aid of a stereo-microscope . Embryos at early–late dicotyledonary stages were cultured on MS medium supplemented with various combinations of 2,4-dichlorophenoxy acetic acid (2,4-D), $N^6$-benzylamino purine (BAP) and thidiazuron (TDZ) either alone or in combination with α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), $N^6$-furfuryladenine (kinetin), abscisic acid (ABA), gibberellic acid ($GA_3$) and casein hydrolysate (CH), for callus induction and morphogenesis.

Four zygotic embryos were placed in one Petri dish with 16 dishes for each treatment. Data were collected as the number of responding zygotic embryos relative to the total number of zygotic embryos cultured. After 5 weeks of culture, the calli were transferred to fresh medium of parental composition for further multiplication and to obtain regeneration of plants from them.

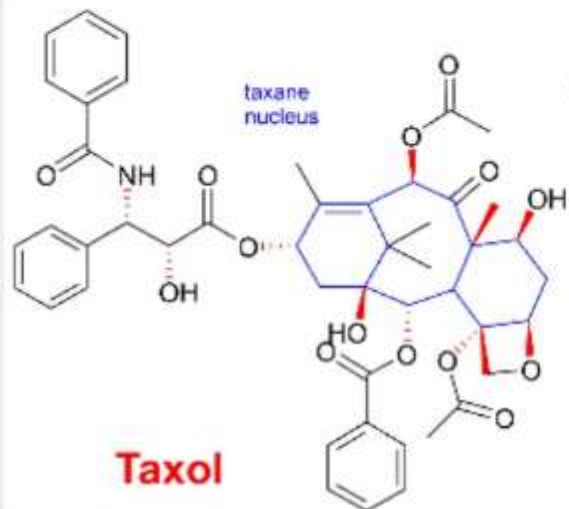
## Secondary Metabolites of Therapeutic Value

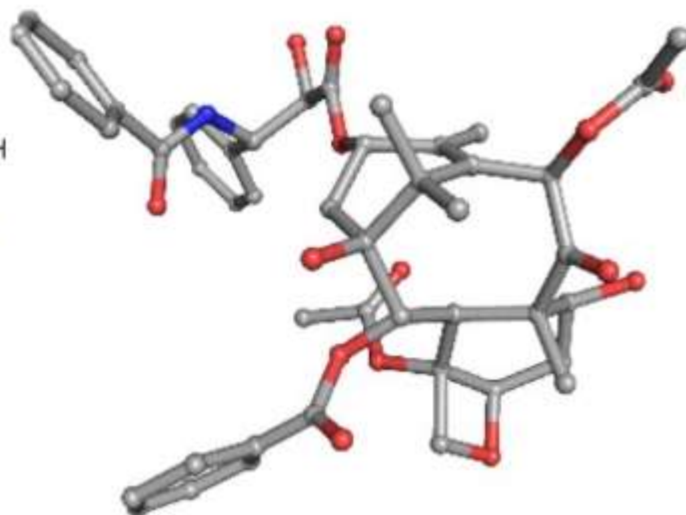
1. **Anticancer drugs e.g. taxol, vinblastine, vincristine, podophyllotoxin and camptothecin**

2. **Cardiovascular drugs e.g. cardenolides/cardiac glycosides (*Digitalis lanata and D. purpurea*).** Addition of progesterone to callus culture of *D. purpurea*, resulted in accumulation of digoxin and digitoxin.

3. **Tonics e.g. ginsenosides (*Panax ginseng*).** Exposure of callus culture to $\gamma$-rays resulted in accumulation of good levels of saponins.

4. **Antispasmodics e.g. furanchromones (*Ammi visnaga*).** Exposure of callus, cell suspension and hairy root cultures to acetylsalicyclic acid, jasmonic acid and silicon dioxide resulted in good levels of furanochromones and pyranocoumarins.

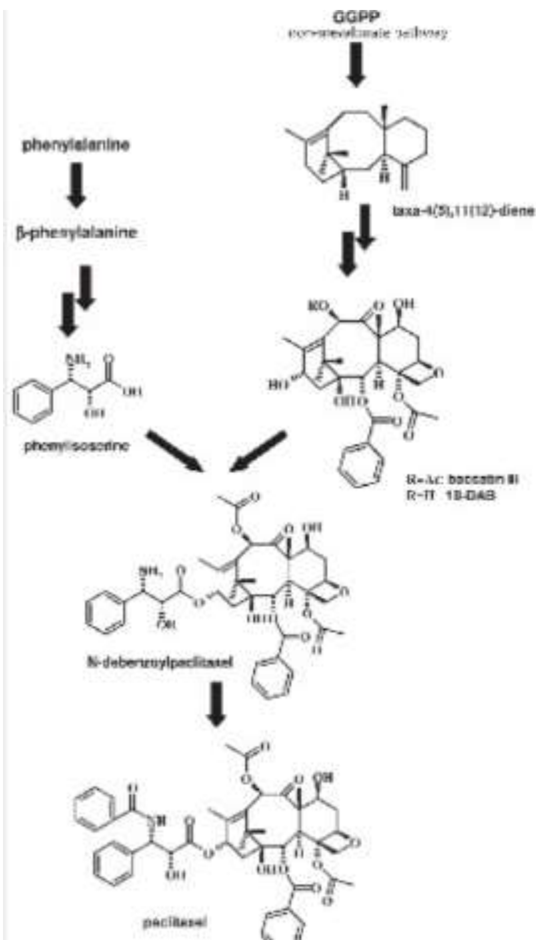


(2a*R*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12b*S*)-9-(((2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl)oxy)-12-(benzoyloxy)-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-3,4,4a,5,6,9,10,11,12,12a-decahydro-1*H*-7,11-methanocyclodeca[3,4]benzo[1,2-*b*]oxete-6,12b(2a*H*)-diyl diacetate

- **Taxol**, a complex diterpene amide, was first isolated from the bark of *Taxus brevifolia* (pacific yew tree) after initial studies at 1962, and its structure was elucidated at **1971**. *Taxus brevifolia* contains low amounts of toxic alkaloids (**taxine A and B**) and reasonable levels of taxol compared to other *Taxus* species.
- Taxol and taxol analogues are the most important drugs for treatment of **drug-refractory ovarian cancer** as well as **lung and breast cancers**.

- *Taxus* species (pacific yew trees) are scarce and thus environmentally protected due to their slow growth (6-10 years to grow).
- The content of taxol is generally very low in Taxus species. The bark of *T. brevifolia* is very thin.
- Semisynthetic approaches for production of taxol from 10-deacetylbaccatin III (more abundant precursor in *T. baccata,*) were initially developed for commercial production (however the supply of 10-deacetylbaccatin III is also limiting, and the overall process is expensive).
- Currently, plant cell culture is the major source for commercially available taxol.




**Taxol biosynthesis**: The pathway comprises four steps:

- Supply of geranylgeranyl diphosphate (GGPP, the universal intermediate of diterpenoids) via a non-mevalonate pathway, as catalysed by **GGPP synthase**.
- Taxane-ring formation as catalysed by **taxadiene synthase**.
- Formation of **baccatin III** (an important intermediate in taxol biosynthesis, **many P450 oxygenases are involved**).
- Introduction and modification of the **phenylisoserine** side chain at C-13 of the taxane ring.

- Production of taxol is enhanced by elicitation with methyl jasmonate (MeJA) and other elicitors, and yields of ~295 mg/L have been reported. There are **two main regulatory steps** in taxol biosynthesis in *Taxus* cell suspension cultures; the taxane ring-formation step and the C-13 side chain-introduction step. MeJA addition upregulates 9 genes in taxol biosynthesis.
- Factors that can be manipulated to increase taxol levels include gas composition ($O_2$, $CO_2$ and ethylene), osmotic pressure, and conditioned medium; bioprocessing strategies, feeding of precursors or sugars.
- Sucrose feeding during stationary phase results in higher cell growth and higher taxol levels from Day 27 Day 42.
- The accumulation of taxol in cells leads to feedback repression and product degradation. Therefore, the *in situ* solvent extraction (two-phase culture) of paclitaxel from suspension cultures is essential for improving productivity. Addition of 10% (v/v) dibutyl-phthalate during the late-log phase improved taxol production and release with minimal inhibitory effect on cell growth.
- Bioreactors of up to 75,000 L are being employed by ESCAgenetic (USA), Phyton (USA), Samyang Genex (Korea), and Phyton Biotech (Germany).
- Overall, combination of the above findings resulted in >100 fold improvement in taxol levels compared to that reported in the first patent concerning taxol production employing suspension cultures. Genetic and metabolic engineering efforts have the potential to further improve the levels of taxol and other valuable products.