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# Plant Cell Cultures - An Alternative and Efficient Source for the Production of Biologically Important Secondary Metabolites

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**Abstract:** Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. Biotechnological applications of plant cell cultures presents the most updated reviews on current techniques in plant culture in the field. The evolving commercial importance of the secondary metabolites has in recent years resulted in a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. In addition to its importance in the discovery of new medicines, plant cell culture technology plays an even more significant role in solving world hunger by developing agricultural crops that provide both higher yield and more resistance to pathogens and adverse environmental and climatic conditions. This paper describes the callus and suspension culture methods that we have established in our laboratory for the production of bioactive secondary metabolites from medicinal plants.

**Keywords**: bioreactors; callus cultures; secondary metabolites; suspension cultures; pharmaceuticals; precursor feeding.

## 1. Introduction

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Bioactive compounds currently extracted from plants are used as food additives, pigments, dyes, insecticides, cosmetics and perfumes and fine chemicals [Balandrin and Klocke, 1988]. These compounds belong to a group collectively known as secondary metabolites. Studies on plant secondary metabolites have been increasing over the last 50 years. The molecules are known to play a major role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals [Ramachandra Rao and Ravishankar, 2002].

In recent years, traditional system of medicine has become a topic of global importance. Although modern medicine may be available in developed countries, herbal medicines [phytopharmaceuticals] have often maintained popularity for historical and cultural reasons.

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Many of the plant species that provide medicinal herbs have been scientifically evaluated for their possible medical applications. It has been mentioned that natural habitats for medicinal plants are disappearing fast and together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals.

Advances in biotechnology particularly methods for culturing plant cell cultures, should provide new means for the commercial processing of even rare plants and the chemicals they provide. These new technologies will extend and enhance the usefulness of plants as renewable resources of valuable chemicals. There has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary metabolites [Dicosmo and Misawa, 1995]. Plant cell culture technologies were introduced at the end of 1960s as a possible tool for both studying and producing plant secondary metabolites. Different strategies using cell cultures systems have been extensively studied with the objective of improving the production of bioactive secondary metabolites. Cell culture systems could be used for the large scale culturing of plant cells from which secondary metabolites can be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. The major advantages of cell cultures includes (i) synthesis of bioactive secondary metabolites is running in controlled environment, independently from climatic and soil conditions; (ii) negative biological influences that affect secondary metabolites production in the nature are eliminated (microorganisms and insects); (iii) it is possible to select cultivars with higher production of secondary metabolites; (iv) with automatization of cell growth control and metabolic processes regulation, cost price can decrease and production increase. The scheme of production of some important plant pharmaceuticals produced in cell cultures has been presented in Table 1. The objectives of many industries are to develop plant cell culture techniques to the stage where they yield secondary products more cheaply than extracting either the whole plant grown under natural conditions or synthesizing the product. Although the production of pharmaceuticals using plant cell cultures have been highlighted, other uses have also been suggested as new route for synthesis, for products from plants difficult to grow, or in short supply, as a source of novel chemicals and as biotransformation systems. It is expected that the use, production of market price and structure would bring some of the other compounds to a commercial scale more rapidly and *in vitro* culture products may see further commercialization. Recent research results indicate that plant cell suspension cells can be used for recombinant protein production under controlled conditions [Fischer et al., 1999]. The aim of the present review is to focus the successful research accomplishments obtained on callus and suspension cultures for production of bioactive secondary metabolites in our research laboratory.

## 2. Accumulation of secondary metabolites in plant cell cultures

For plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells [Berlin and Sasse, 1985]. Careful selection of productive cells and cultural conditions resulted in accumulation of several products in higher levels in cultured cells. In order to obtain yields in high concentrations for commercial exploitation, efforts have focused on the stimulation of biosynthetic activities of cultured cells using various methods (Ramachandra Rao, 2000; Dixon, 1999; Ravishankar and Venkataraman, 1993; Buite-

laar and Tramper, 1992).

**Table 1.** Bioactive secondary metabolites from plant cell cultures

| Plant name | Active ingredient | Culture type | Reference |
|---|---|---|---|
| *Agave amaniensis* | Saponins | Callus | Andrijany et al., 1999. |
| *Ailanthus altissima* | Alkaloids | Suspension | Anderson et al., 1987. |
| *Ailanthus altissima* | Canthinone alkaloids | Suspension | Anderson et al., 1986. |
| *Allium sativum* L. | Alliin | Callus | Malpathak and David , 1986. |
| *Aloe saponaria* | Tetrahydroanthracene glucosides | Suspension | Yagi et al., 1983. |
| *Ambrosia tenuifolia* | Altamisine | Callus | Goleniowski and Trippi , 1999. |
| *Anchusa officinalis* | Rosmarinic acid | Suspension | De-Eknamkul and Ellis, 1985. |
| *Brucea javanica* (L.) Merr. | Canthinone alkaloids | Suspension | Liu et al., 1990. |
| *Bupleurum falcatum* | Saikosaponins | Callus | Wang and Huang, 1982. |
| *Camellia Sinensis* | Theamine, γ-glutamyl derivatives | Suspension | Orihara and Furuya, 1990. |
| *Canavalia ensiformis* | L-Canavanine | Callus | Ramirez et al., 1992. |
| *Capsicum annuum* L. | Capsaicin | Suspension | Johnson et al., 1990. |
| *Cassia acutifolia* | Anthraquinones | Suspension | Nazif et al., 2000. |
| *Catharanthus roseus* | Indole alkaloids | Suspension | Moreno et al., 1993. |
| *Catharanthus roseus* | Catharanthine | Suspension | Zhao et al., 2001. |
| *Choisya ternata* | Furoquinoline alkaloids | Suspension | Sejourne et al., 1981. |
| *Chrysanthemum cinerariaefolium* | Pyrethrins | Callus | Rajasekaran et al., 1991. |
| *Chrysanthemum cinerariaefolium* | Chrysanthemic acid and pyrethrins | Suspension | Kueh et al., 1985. |
| *Cinchona* L. | Alkaloids | Suspension | Koblitz et al., 1983. |
| *Cinchona robusta* | Robustaquinones | Suspension | Schripsema et al., 1999. |
| *Cinchona* spe. | Anthraquinones | Suspension | Wijnsma et al., 1985. |
| *Cinchona succirubra* | Anthraquinones | Suspension | Khouri et al., 1986. |
| *Citrus* sp. | Naringin, Limonin | Callus | Barthe et al., 1987. |
| *Coffea arabica* L. | Caffeine | Callus | Waller et al., 1983. |
| *Cornus kousa* | Polyphenols | Suspension | Ishimaru et al., 1993. |
| *Corydalis ophiocarpa* | Isoquinoline alkaloids | Callus | Iwasa and Takao, 1982. |
| *Croton sublyratus* Kurz | Plaunotol | Callus | Morimoto and Murai, 1989. |
| *Cruciata glabra* | Anthraquinones | Suspension | Dornenburg and Knorr, 1996. |
| *Cryptolepis buchanani* Roem. & Schult | Cryptosin | Callus | Venkateswara et al., 1987. |
| *Digitalis purpurea* L. | Cardenolides | Suspension | Hagimori et al., 1982 |
| *Dioscorea deltoidea* | Diosgenin | Suspension | Heble and Staba, 1980. |
| *Dioscorea doryophora* Hance | Diosgenin | Suspension | Huang et al., 1993. |
| *Duboisia leichhardtii* | Tropane alkaloids | Callus | Yamada and Endo, 1984. |
| *Ephedra s*pp. | L- Ephedrine D-pseudoephedrine | Suspension | O'Dowd et al., 1993. |

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| *Eriobotrya japonica* | Triterpenes | Callus | Taniguchi et al., 2002. |
| *Eucalyptus tereticornis* SM. | Sterols and Phenolic compounds | Callus | Venkateswara et al., 1986. |
| *Eucommia ulmoides* | Chlorogenic acid | Suspension | Wang et al., 2003. |
| *Fumaria capreolata* | Isoquinoline alkaloids | Suspension | Tanahashi and Zenk, 1985. |
| *Gentiana* sp. | Secoiridoid glucosides | Callus | Skrzypczak et al., 1993. |
| *Ginkgo biloba* | Ginkgolide A | Suspension | Carrier et al., 1991. |
| *Glehnia littoralis* | Furanocoumarin | Suspension | Kitamura et al., 1998. |
| *Glycyrrhiza echinata* | Flavanoids | Callus | Ayabe et al., 1986. |
| *Glycyrrhiza glabra* var. *glandulifera* | Triterpenes | Callus | Ayabe et al., 1990. |
| *Hyoscyamus niger* | Tropane alkaloids | Callus | Yamada and Hashimoto, 1982. |
| *Isoplexis isabellina* | Anthraquinones | Suspension | Arrebola et al., 1999. |
| *Linum flavum* L. | 5-Methoxypodophyllotoxin | Suspension | Uden et al., 1990. |
| *Lithospermum erythrorhizon* | Shikonin derivatives | Suspension | Fujita et al., 1981. |
| *Lithospermum erythrorhizon* | Shikonin derivatives | Suspension | Fukui et al., 1990. |
| *Lycium chinense* | Cerebroside | Suspension | Jang et al., 1998. |
| *Morinda citrifolia* | Anthraquinones | Suspension | Zenk et al ., 1975. |
| *Morinda citrifolia* | Anthraquinones | Suspension | Bassetti et al., 1995. |
| *Mucuna pruriens* | L-DOPA | Suspension | Wichers et al., 1993. |
| *Mucuna pruriens* | L-DOPA | Callus | Brain K. R., 1976. |
| *Nandina domestica* | Alkaloids | Callus | Ikuta and Itokawa, 1988. |
| *Nicotiana rustica* | Alkaloids | Callus | Tabata and Hiraoka, 1976. |
| *Nicotiana tabacum* L. | Nicotine | Suspension | Mantell et al., 1983. |
| *Nothapodytes foetida* | Camptothecin | Callus | Thengane et al., 2003. |
| *Ophiorrhiza pumila* | Camptothecin related alkaloids | Callus | Kitajima et al., 1998. |
| *Panax ginseng* | Saponins and Sapogenins | Callus | Furuya et al., 1973. |
| *Panax notoginseng* | Ginsenosides | Suspension | Zhong and Zhu, 1995. |
| *Papaver bracteatum* | Thebaine | Callus | Day et al., 1986. |
| *Papaver somniferum* L. | Alkaloids | Callus | Furuya et al., 1972. |
| *Papaver somniferum* | Morphine , Codeine | Suspension | Siah and Doran, 1991. |
| *Peganum harmala* L. | β-Carboline alkaloids | Suspension | Sasse et al., 1982. |
| *Phytolacca americana* | Betacyanin | Suspension | Sakuta et al., 1987. |
| *Picrasma quassioides* Bennett | Quassin | Suspension | Scragg and Allan, 1986. |
| *Podophyllum hexandrum* royle | Podophyllotoxin | Suspension | Uden et al., 1989. Chattopadhyay et al., 2002. |
| *Polygala amarella* | Saponins | Callus | Desbene et al., 1999. |
| *Polygonum hydropiper* | Flavanoids | Suspension | Nakao et al., 1999. |
| *Portulaca grandiflora* | Betacyanin | Callus | Schroder and Bohm, 1984. |
| *Ptelea trifoliata* L. | Dihydrofuro [2,3-b] quinolinium alkaloids | Callus | Petit-Paly et al., 1987. |
| *Rauwolfia sellowii* | Alkaloids | Suspension | Rech et al., 1998. |
| *Rauwolfia serpentina* Benth. | Reserpine | Suspension | Yamamoto and Yamada, 1986. |

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| *Rauvolfia serpentina* x *Rhazya stricta* Hybrid plant | 3-Oxo-rhazinilam | Callus | Gerasimenko et al., 2001. |
| *Ruta s*p. | Acridone and Furoquinoline alkaloids and coumarins | Callus | Baumert et al., 1992. |
| *Salvia fruticosa* | Rosmarinic acid | Callus & suspension | Karam et al., 2003 |
| *Salvia miltiorrhiza* | Lithospermic acid B and Rosmarinic acid | Callus | Morimoto et al., 1994. |
| *Salvia miltiorrhiza* | Cryptotanshinone | Suspension | Miyasaka et al., 1989. |
| *Sapium sebiferum* | Tannin | Callus & suspension | Neera and Ishimaru, 1992. |
| *Scopolia parviflora* | Alkaloids | Callus | Tabata et al., 1972. |
| *Scutellaria columnae* | Phenolics | Callus | Stojakowska and Kisiel, 1999. |
| *Solanum chrysotrichum* (Schldl.) | Spirostanol saponin | Suspension | Villarreal et al., 1997. |
| *Solanum laciniatum* Ait | Solasodine | Suspension | Chandler and Dodds, 1983. |
| *Solanum paludosum* | Solamargine | Suspension | Badaoui et al., 1996. |
| *Stizolobium hassjoo* | L-DOPA | Suspension | Huang et al., 2002. |
| *Tabernaemontana divaricata* | Alkaloids | Suspension | Sierra et al., 1992. |
| *Taxus* spp. | Taxol | Suspension | Wu et al., 2001. |
| *Taxus baccata* | Taxol, baccatin III | Suspension | Cusido et al., 1999. |
| *Taxus cuspidata* | Taxoids | Suspension | Ketchum et al., 2003. |
| *Tecoma sambucifolium* | Phenylpropanoid glycosides | Callus | Pletsch et al., 1993 |
| *Thalictrum minus* | Berberin | Suspension | Kobayashi et al., 1987. |
| *Thalictrum minus* | Berberin | Suspension | Nakagawa et al., 1986. |
| *Torreya nucifera* var. *radicans* | Diterpenoids | Suspension | Orihara et al., 2002. |
| *Trigonella foenumgraecum* | Saponins | Suspension | Brain and Williams, 1983. |

Culture productivity is critical to the practical application of plant cell culture technology to production of plant-specific bioactive metabolites. Until now, various strategies have been developed to improve the production of secondary metabolites using plant cell cultures. The tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions. That means maximization of the production and accumulation of secondary metabolites by plant tissue cultured cells requires (i) manipulating the parameters of the environment and medium, (ii) selecting high yielding cell clones, (iii) precursor feeding, and (iv) elicitation.

**Optimization of cultural conditions**: number of chemical and physical factors like media components, phytohormones, pH, temperature, aeration, agitation, light affecting production of secondary metabolites has been extensively studied (Lee and Shuler, 2000; Wang et al., 1999; Fett-Neto et al., 1995. Goleniowski and Trippi, 1999). Several products were found to be accumulating in cultured cells at a higher level than those in native plants through optimization of cultural conditions. Manipulation of physical aspects and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity. For example, ginsenosides by *Panax ginseng* [Choi et al., 1994; Furuya et al., 1984; Franklin and

Dixon, 1994; Furuya, 1988;], rosmarinic acid by *Coleus bluemei* [Ulbrich et al., 1985], shikonin by *Lithospermum erythrorhizon* [Takahashi and Fujita, 1991], ubiquinone-10 by *Nicotiana tabacum* [Fontanel and Tabata, 1987], berberin by *Coptis japonica* [Matsubara et al., 1989], were accumulated in much higher levels in cultured cells than in the intact plants.

**Selection of high-producing strains:** plant cell cultures represent a heterogeneous population in which physiological characteristics of individual plant cells are different. Synthesis of several products in high amounts using selection and screening of plant cell cultures have been described by Berlin and Sasse [1985]. Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. A strain of *Euphorbia milli* accumulated about 7-fold the level of anthocyanins produced by the parent culture after 24 selections [Yamamoto et al., 1982]. Selection can be easily achieved if the product of interest is a pigment (Fujita et al., 1984). Cell cloning using cell aggregates of *Coptis japonica* [Yamada and Sato, 1981], and obtained strain, which grew faster and produced a higher amount of berberin and cultivated the strain in a 14 L bioreactor. Selected cell line increased growth about 6-fold in 3 weeks and the highest amount of alkaloid was produced 1.2 g/L of the medium and the strain was very stable, producing a high level of berberin even after 27 generations. Increased capsaicin and rosmarinic acid in PEP cell lines of *Capsicum annuum* were reported (Salgado-Garciglia and Ochoa-Alejo, 1990). Selective agents such as 5-methyltryptophan, glyphosate and biotin have also been studied to select high-yielding cell lines (Amrhein et al., 1985; Watanabe et al., 1982; Widholm, 1974).

**Precursor feeding**: exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. This approach is useful when the precursors are inexpensive. The concept is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases (Silvestrini et al., 2002; Moreno et al., 1993; Whitmer et al., 1998). For example, amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids etc. Addition of phenylalanine to *Salvia officinalis* cell suspension cultures stimulated the production of rosmarinic acid [Ellis and Towers, 1970]. Addition of the same precursor resulted stimulation of taxol production in *Taxus* cultures [Fett-Neto et al., 1993 and 1994]. Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in increase in vanillin accumulation (Romagnoli and Knorr, 1988). Furthermore, addition of leucine, led to enhancement of volatile monoterpenes in cultures of *Perilla frutiscens*, where as addition of geraniol to rose cell cultures led to accumulation of nerol and citronellol (Mulder-Krieger et al., 1988).

**Elicitation**: plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens. Elicitors are signals triggering the formation of secondary metabolites. Use of elicitors of plant defense mechanisms, i.e. elicitation, has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites (Roberts and Shuler, 1997). Biotic and abiotic elicitors which are classified on their origin are used to stimulate secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations (Barz et al., 1988; Eilert, 1987; DiCosmo and Tallevi, 1985). Production of many valuable secondary metabolites using various elicitors were reported

(Wang and Zhong, 2002a, 2002b; Dong and Zhong, 2001; Hu et al., 2001; Lee and Shuler, 2000).

## 3. Cell culture protocols for production of important secondary metabolites established in our laboratory

Most applications of plant cell cultures in biotechnology are aimed at the production of bioactive secondary metabolites. This has included production of taxol, morphine and codeine, ginsenosides, L-DOPA, berberine, diosgenin, capsaicin, podophyllotoxin, shikonin derivatives, ajmalicine, vincristine and vinblastine [Dicosmo and Misawa, 1995]. Since cell suspension cultures are preferred for large-scale production due to its rapid growth cycles they have been used for generating large amounts of cells for quantitative or qualitative analysis of growth responses and metabolism of novel chemicals. In our laboratory, we focus on the production of some important pharmaceuticals in plant cell cultures. We have successfully established cell cultures for production of taxol from *Taxus mairei*, imperatorin from *Angelica dahurica*, diosgenin from *Dioscorea doryophora*, gentipicroside and swertiamarin from *Gentiana*, cryptotanshinone from *Salvia miltiorrhiza* and the results were described in the present review.

### 3.1. Case study 1: Production of taxol from *Taxus mairei* by cell suspension cultures

As early as 1969, Wani and his colleagues discovered a novel anticancer diterpene amide, "taxol" from the Pacific yew (*Taxus brevifolia*) extract [Wani et al, 1971]. In 1983, taxol was approved to enter phase I clinical trials for ovarian cancer by the Food & Drug Administradon (FDA) in the USA. It has been approved for clinical treatment of ovarian and breast cancer by the FDA, and it also had significant activity in the treatment of patients with malignant melanoma, lung cancer, and other solid tumors [Wickremesinhe and Arteca, 1993 and 1994]. Taxol is considered as the prototype of a new class of cancer chemotherapeutic agents [Cragg et al., 1993].

However the supply of taxol for clinical use is limited. It depends on extraction from yew trees, and the bark is the only commercial source. The thin bark of yew tree contains 0.001% of taxol by dry weight basis. A century old tree yields an average of 3 kg of bark, corresponding to 300 mg of taxol, which is approximately a single dose in the course of a cancer treatment. Because of the scarcity of the slow growing trees and the relatively low content of taxol [Cragg et al., 1993], alternative sources are needed to meet the increasing demand for the drug. The total synthesis of taxol on an industrial-scale seems economically unrealistic due to the complexity of the chemical structure of this molecule [Holton et al., 1994; Nicolaou et al., 1994]. The plant cell culture of *Taxus* spp. is considered as one of the approach available to provide a stable supply of taxol and related taxane derivatives [Slichenmyer and Von Horf, 1991].

For exploiting the source of taxol, we collected different tissues of *Taxus mairei*, a species found in Taiwan at an altitude of about 2000m above the sea level. The extracts of bark and leaf tissues were analyzed by using HPLC for the content of taxol and taxol related compounds. The HPLC analysis revealed that the amounts of taxol and taxol related compounds varies in individual plants, and the principle components such as docetaxel, baccatin III, and 10-deacetylbaccatin in leaf extract were higher than those in bark extracts [Lee et al., 1995]. *Taxus mairei* calli were induced from needle and stem explants on B5 medium [Gamborg's et al., 1968] supplemented with 2 mg/l 2,4-D or NAA. Different cell lines were established using stem and needle-derived callus. One of the cell lines, after precursor feeding and 6 weeks of incubation, produced 200 mg taxol per liter cell suspension cultures.

### 3.2. Case study 2: Formation of imperatorin from *Angelica dahurica* var. *formosana* by cell suspension cultures

*Angelica dahurica* var. *formosana* commonly known as "Bai-Zhi" in Chinese is a valuable medicinal herb used in the treatment of headache and psoriasis in China [Zhou, 1980]. The constituent imperatorin has been suggested as the major active ingredient for curing the skin disease [Zhou et al., 1988]. *Angelica dahurica* var. *formosana* is a perennial and indigenous plant in Taiwan [Chen et al., 1994].

As the production of Bai-Zhi by conventional cultural method is far short to meet the demand, cell suspension culture methods for production of imperatorin was therefore emphasized. For establishing a rapidly growing and finely dispersed cell suspension culture, the best medium composition was 1/2 strength of MS medium [Murashige and Skoog's, 1962] supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and 3% sucrose. Al the cultures were routinely sub cultured at an interval of 14 days.

In a growth cycle of the suspension cells, the production of imperatorin was maximum between the $10^{th}$ and $14^{th}$ day. Growth conditions, especially the nutritional factors optimal for imperatorin production were also investigated. It was found that MS basal media was the best among the media tested on imperatorin production. Influence of auxins on imperatorin production were investigated. Deletion of auxins from the medium was beneficial to imperatorin production and addition of BA (0.5-1 mg/L) promoted synthesis of imperatorin in suspension cells.

The effects of the ratio of ammonium nitrate to nitrate in the medium had also been studied. A moderate ammonium nitrate to nitrate ratio (2:1) increased the amount of imperatorin production. Increasing phosphate concentration (1 mM to 2 mM ) promoted imperatorin production. Glucose was found to be bettor carbon source than sucrose and fructose in terms of their effects on imperatorin production. The possible stimulating effect of elicitors on imperatorin synthesis were studied. Addition of vanadyl sulphate to cell suspension cultures enhance the accumulation of imperatorin, depending on its concentration and the growth stage of the cells. The most significant effect was recorded when vanadyl sulphate at the concentration of 30 mg/L was added to the medium on the $10^{th}$ day of the culture. A rapid and drastic increase in imperatorin synthesis was observed by adding 20 g/L of adsorbent Amberlite XAD-7 to suspension cells on the $10^{th}$ day of culture. The quantity of imperatorin produced by this treatment (460 $\mu$ g/gd. w.) was 140 folds higher than that produced by the check treatment [Tsay, 1999; Tsay et al., 1994]. These results showed that the cultured cells possessed the biosynthetic potential of the intact plants from which they were derived. The biosynthetic potential could be further facilitated by the addition of proper stimulants to the culture medium.

### 3.3. Case study 3: Diosgenin production from *Dioscorea doryophora* by cell suspension cultures

*Dioscorea* spp. (Dioscoreaceae) is frequently used as tonic in Chinese traditional medicine. *Dioscorea doryophora* Hance, tubers are in high demand as it is used not only as crude drug but also as food. The most active ingredient discovered in the tuber is diosgenin, which can be used as a precursor for many important medicinal steroids such as prednisolone, dexamethasone, norethisterone and metenolone etc. [Tsukamoto et al., 1936].

The purpose of the present study was to establish the cell suspension cultures of *Dioscorea doryophora* Hance for the production of diosgenin, which can be used as an alternative source for the synthesis of steroids [Yeh et al., 1994]. Callus was successfully induced on MS basal medium supplemented with 2.0 mg/L 2, 4-D and 0.2 mg/L BA. Cell suspen-

sion cultures was successfully established by subculturing callus into a liquid medium containing MS basal medium supplemented with 0.2 mg/ L 2, 4-D. Sucrose concentration optimal for diosgenin production was investigated. Although 6% sucrose was an optimum concentration for the growth and increasing dry weight of the cell suspension culture, 3% sucrose gave better results of the diosgenin production. Differences in the quality and diosgenin content of the induced callus were observed among the six source organs. Analysis by HPLC revealed that both stem-node and microtuber derived suspension cells contained diosgenin. The content of microtuber derived cell suspension culture contains 3.2% diosgenin per gram dry weight, where as, the stem-node derived cultures contains only 0.3%. Callus from microtuber showed yellowish color and granular in appearance and contained the highest diosgenin content (3.3-3.5%). As the amount of diosgenin obtained from tuber derived cell suspension is high and comparable with that found in the intact tuber [Chen, 1985], cell suspension culture could be used for production of diosgenin.

### 3.4. Case study 4: Establishment of *Gentiana davidii* var. *formosana* (Hayata) T. N.Ho cell suspension cultures

*Gentiana davidii* var. *formosana* (Hayata) T. N. Ho (Gentianaceae), commonly known as Long-dan in Chinese is a perennial herb indigenous to Taiwan. Regular consumption of Long-dan has been believed to enhance memory, prevent obesity and aging, and protect the liver by removing toxic metabolites [Lou and Chin, 1996 and Zheng et al., 1997]. The entire dried herb, collected from the wild habitat, is used as a crude drug in traditional Chinese medicine in Taiwan. Bitter principles of Gentianaceae constitute many pharmacologically important compounds, which justify the use of most species of this family in traditional medicine or for the preparation of bitter tonic [Rodriguez et al., 1996]. Secoiridoid glycosides are the main compounds with medicinal properties in roots of *Gentiana* species [Skrzypczak et al., 1993].

We have optimized the conditions for establishment of cell suspension cultures of *G. davidii* for the production of gentipicroside and swertiamarin, the two pharmacologically important compounds. Callus was initiated by culturing stem explants of *G. davidii* var. *formosa* on MS basal medium supplemented with 0.2 mg/L kinetin and 1.0 mg/L $\alpha$ -naphthaleneacetic acid (NAA). Fast growing cell suspension cultures were established by subculturing the callus in MS basal medium supplemented with 0.2 mg/L kinetin and 3% sucrose [Chueh et al., 2000].

Optimal cell growth was obtained when callus cultured in 25 mL liquid MS basal medium supplemented with 0.2 mg/L kinetin and 3% sucrose, pH between 4.2-5.2, incubated in light 2.33 $\mu$ E $m^{-2}$ $s^{-1}$ at 25 $\pm$1℃, under 80-100 rev/min shaker speed. The maximum content of the two principles, swertiamarin and gentipicroside, in cell suspension were obtained after 12 and 24 days of culture respectively. Using this standard protocol, it may be possible to study the effect of precursor feeding on the content of active principles.

### 3.5. Case study 5: Production of cryptotanshinone from callus cultures of *Salvia miltiorrhiza* Bunge.

*Salvia* is an important genus consisting of ca. 900 species in the family Lamiaceae and some species of *Salvia* have been cultivated worldwide for use in folk medicine. Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge, is one of the most popular Chinese medicines and widely used for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood and tranquilizing the mind and cooling blood to relieve carbuncles [Verpoorte, 1998]. The major active principles of the organic extract of Dan-shen are tanshinones, the quinoid

diterpenes [Bruneton, 1995]. Since Dan-shen preparations constitute a basis for considerable commercial activity, there is a continued interest in development of biotechnology-based approaches to the production of tanshinones [Hu and Alfermann, 1993; Miyasaka et al., 1989 and Shimomura et al., 1991]. In our continuing study of the application of tissue culture of medicinal plants to the production of bioactive secondary metabolites, we have adopted an approach to the production of cryptotanshinone from *S. miltiorrhiza* through callus cultures.

In this experiment effect of $N^6$- benzyladenine (BA) on cryptotanshinone formation in callus cultures of *Salvia miltiorrhiza* was examined. Primary callus was induced by culturing leaf explants on Murashige and Skoog's (MS) basal medium supplemented with 1.0 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) in darkness. The callus proliferated further on MS basal medium containing 1.0 mg/L 2,4-D and 0.5 mg/L BA and was analyzed for cryptotanshinone by high performance liquid chromatography (HPLC). The HPLC results indicated that it contained small amounts of cryptotanshinone (0.26 ± 0.05 mg/g dry wt.). Omission of 2,4-D from the medium resulted in a marked increase in the content of cryptotanshinone in callus. The HPLC analysis revealed that the content of cryptotanshinone in the callus cultured on the MS basal medium supplemented with 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L of BA was significantly higher than the marketed crude drug (processed underground parts of *S. miltiorrhiza*). Maximum yield of cryptotanshinone (4.59 ± 0.09 mg/g dry wt.) was observed in the callus cultured on MS basal medium supplemented with 0.2 mg/L BA for sixty days [Wu et al., 2003].

## 4. Conclusions

The use of plant cell culture for the production of chemicals and pharmaceuticals has made great strides building on advances in plant science. The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of product. The increased appeal of natural products for medicinal purposes coupled with the low product yields and supply concerns of plant harvesation has renewed interest in large-scale plant cell culture technology. Knowledge of biosynthetic pathways of desired compounds in plants as well as in cultures is often still in its infancy, and consequently, strategies are needed to develop an information based on a cellular and molecular level. Because of the complex and incompletely understood nature of plant cells in *in vitro* cultures, case-by-case studies have been used to explain the problems occurring in the production of secondary metabolites from cultured plant cells. These results shows that plant cell culture systems have potential for commercial exploitation of secondary metabolites. The introduction of the techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step towards making cell cultures more generally applicable to the commercial production of secondary metabolites.

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