

# Tissue Culture Technology of Chinese Medicinal Plant Resources in Taiwan and their Sustainable Utilization

Hsin-Sheng Tsay\* and Dinesh Chandra Agrawal

Graduate Institute of Biotechnology,  
Chaoyang University of Technology,  
168, Jifong E. Road, Wufong, Taichung 413, Taiwan

**Abstract:** There has been a renewed interest in the use of herbal medicines throughout the world due to toxicities and health hazards associated with synthetic drugs and antibiotics. About 85% of traditional medicines involve the use of plant extracts. However, a large number of medicinal plants still remain to be investigated for their possible pharmacological values. Majority of the pharmaceutical industries harness wild growing plant populations, for the supply of raw materials for extraction of medicinally important compounds. Many of the medicinal plants are severely threatened owing to illicit and indiscriminate collection and destruction of natural habitats. Advanced biotechnological methods of culturing plant cells and tissues provide alternative means for rapid propagation and conservation of rare and endangered and/or commercially important medicinal plants. The present paper reviews the work carried out in our group in Taiwan on *in vitro* propagation of *Pinellia ternata*, somatic embryogenesis in *Corydalis yanhusuo* and production of secondary plant metabolites from callus of *Salvia miltiorrhiza* and cell suspension cultures of *Dioscorea doryophora*.

**Keywords:** *Corydalis yanhusuo*; *Dioscorea doryophora*; *Pinellia ternata*; *Salvia miltiorrhiza*; secondary plant metabolites.

## 1. Introduction

Millions of people in the third world countries prefer herbal medicines because they believe in them and regard as “their” medicines in contrast to western allopathic prescriptions. Over the last 2500 years, there have been very strong traditional systems of medicine such as Chinese, Ayurvedic, and the Unani, born and practiced, more in eastern continent. These traditions are still flourishing, since; approximately 80% of the people in the developing countries rely on these systems of medicine for their primary health care needs. Now, even in western countries too, there is

increased awareness about herbal medicines, largely due to powerful synthetic agents used in the western medicine which exert more unwanted side effects. About 85% of traditional medicines involve the use of plant extracts (Vieira and Skorupa, 1993). Many plant species, possessing medicinally important compounds, are disappearing at an alarming rate due to destruction of its natural habitats owing to rapid agricultural development, urbanization, indiscriminate deforestation and uncontrolled collection of plant materials.

The central mountain range of Taiwan is home of many highly valued medicinal herbs. Like many other parts in the world, in Taiwan

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\* Corresponding author; e-mail: [hstsay@mail.cyut.edu.tw](mailto:hstsay@mail.cyut.edu.tw)

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too, medicinal herbs are being indiscriminately collected in large quantities from the wild to meet the ever-increasing demand for traditional crude drugs. Plant Biotechnology can play a significant role in increasing plant population of locally available medicinally important plant species and in its conservation and maintenance of biodiversity. The National Science Council (NSC) of Taiwan has been promoting research on traditional Chinese medicinal plants since 1988. The main objectives of these research programme are: (i) to collect information about important and rare traditional medicinal herbs, (ii) to develop simple methods of identification of medicinal herbs, (iii) to develop methods for mass propagation of medicinal herbs through tissue culture, (iv) to study active principles and pharmacology for their safer use, and (v) to promote export of traditional medicinal herbs. At Taiwan Agricultural Research Institute (TARI) and Chaoyang University of Technology (CYUT), work has been carried out on mass propagation of several important native and traditional Chinese medicinal herbs. We have successfully propagated many medicinally important species through shoot morphogenesis and / or somatic embryogenesis (Nalawade et al., 2003; Nalawade and Tsay, 2004). Also, we have been successful in establishing callus and cell suspension culture of several plant species and extraction of medicinally important compounds (Mulabagal et al., 2004a; b). Present communication describes the work carried out at the TARI and CYUT on *in vitro* propagation of *Pinellia ternata*, somatic embryogenesis in *Corydalis yanhusuo* and production of secondary plant metabolites from callus of *Salvia miltiorrhiza* and cell suspension cultures of *Dioscorea doryophora*.

### 2.1. Case study 1: shoot morphogenesis: *In vitro* propagation of *Pinellia ternate*

*Pinellia ternata* belonging to family Araceae grows wildy in Japan and China. It

is a perennial herb. Tubers of *P. ternata* contain homogentisic acid, its glucoside, 3,4-dihydroxybenzaldehyde, its diglucoside and ephedrine (Shoyama et al., 1983a, b). The species has been used in Chinese medicine to prevent vomiting, and for analgesic and sedative effects. The traditional Japanese herbal (Kampo) medicine “Sho-seiryu-to,” being used for the treatment of cold syndromes (Nagai and Yamada, 1994) contains tubers of *P. ternata* as one of the components. Pinellic acid from the tubers had shown oral adjuvant activity for nasal influenza HA vaccine (Nagai et al., 2002). Presently, the species is not under cultivation owing to the limited planting materials in wild, hence tissue culture studies were carried out with a view to establish mass propagation method of this important medicinal plant.

An efficient method of plant regeneration via., adventitious buds or protocorm-like body formation directly from the bulbils, leaf-blade, and petiole explants without intervening callus was developed by Tsay et al., (1989). The explants developed adventitious buds when cultured on MS basal medium supplemented with BA (1-5 mg/l<sup>-1</sup>) and NAA (0.0 - 0.2 mg/l<sup>-1</sup>). The maximum number of adventitious buds (73 per explant ) was induced from protocorm-like body cultured on the MS basal medium supplemented with BA alone without any NAA (Table 1). It was observed that incorporation of NAA in the medium at all levels of BA had adverse effect on the proliferation rate. However, a different trend was observed with adventitious bud as an explant. At higher levels of BA (5.0 mg/l<sup>-1</sup>), proliferation rate was drastically improved on inclusion of NAA in the proliferation medium (Table 1).

The regeneration efficiency varied with the explant type. Maximum response was observed in bulbils, followed by leaf-blades and petiole explants. The protocorm-like bodies induced on the medium containing BA and NAA multiplied at a prolific rate when cut into parts and transferred to a liquid MS basal

medium supplemented with BA (1-15 mg/l<sup>-1</sup>) and NAA (0.2 mg/l<sup>-1</sup>) or 2,4-D (0.2 mg/l<sup>-1</sup>). The regenerative potential of the cultures could be maintained by culturing them alternately in solid and liquid media (Figure 1A-C). Plant regeneration from protocorm-like bodies was observed after continuous culture in liquid medium (Figure 1A). Rooting was achieved in half strength MS basal medium supplemented with NAA (1.0 mg/l<sup>-1</sup>). A high survival rate of plants (96%) was achieved on their transfer to a mixture of vermiculite:loam soil:peat moss potting substrates. No morphological abnormalities in tissue culture raised plants were observed when compared to plants grown in field. By using this *in vitro* procedure, it is estimated that about  $1.7 \times 10^{27}$  plantlets from a single bulbil can be produced in one year.

**Table 1.** Effect of BA and NAA on shoot bud proliferation in adventitious bud and protocorm-like bodies of *P. ternate*\*

BA NAA Mg/l <sup>-1</sup>	Number of buds proliferated per protocorm-like body	Number of buds proliferated per adventitious bud
1   0	55.5 ± 6.3	15.0 ± 3.1
1   0.2	46.0 ± 5.7	12.1 ± 2.0
2   0	73.0 ± 6.8	21.8 ± 3.6
2   0.2	60.2 ± 6.1	23.0 ± 3.4
5   0	70.0 ± 7.2	11.7 ± 2.6
5   0.2	56.1 ± 5.9	21.5 ± 2.9

\* Culture medium: MS basal medium with agar (1%), sucrose (3%).  
Incubation period: 30 days; No. of explants per treatment: 50.

## 2.2. Case study 2: somatic embryogenesis: *In vitro* plant regeneration in *Corydalis* *yanhusuo* via somatic embryogenesis.

The genus *Corydalis* belonging to family Fumariaceae or Papaveraceae, consisting of about 320 species, is widely distributed in the northern hemisphere. About 70 species are

used in traditional herbal remedies in China, Japan and Korea (Kamigauchi and Iwasa, 1994). Rhizoma *Corydalis*, the dried and pulverized tuber of *Corydalis* is used in the treatment of gastric and duodenal ulcer, cardiac arrhythmia disease, and several other ailments (Kamigauchi and Iwasa, 1994). *Corydalis* tubers contain many pharmacologically active alkaloids. Some of the important alkaloids are: *d*-corydaline, *dl*-tetrahydropalmitine, and corydalis H, I, J, K, L (Huang, 1993). *Corydalis yanhusuo* W. T. Wang (syn. *Corydalis turtshaninonii* Bess. f. *yanhusuo* Y.H. Chou et C. C. Hsu), a perennial herb up to 20 cm tall, is cultivated in mainland China as an annual crop by using tubers. The problem with tubers is that these are often infested by fungal diseases; mainly downy mildew (Gao et al., 1991). Infected tubers if planted in soil, result not only into 30-50% loss in yield (Gao et al., 1991), but also affect the quality of the crude drug. Thus, it was essential to develop pathogen-free high quality planting material to boost the production.

A method has been developed for regeneration of complete plants via., somatic embryogenesis in *C. yanhusuo* using tuber-derived callus (Sagare et al., 2000) (Fig. 2A-F). Primary callus was induced on culture of tuber pieces (5×5×2 mm) on MS basal medium supplemented with BA (2.0 mg/l<sup>-1</sup>) and NAA (0.5 mg/l<sup>-1</sup>) and on incubation in complete dark (Fig. 2A). On transfer of this primary callus to MS basal medium supplemented with different concentrations of cytokinins (BA, kinetin and zeatin), and incubation of cultures in light, induction of somatic embryos was observed within 2 weeks. The embryos progressed through the globular, late-globular, heart, early cotyledonary, and cotyledonary stages. After five week, somatic embryos showed development of cotyledonary leaves. For the development of roots, embryos with well-developed cotyledonary leaves were transferred to half-strength liquid MS medium supplemented with zeatin ri-

boside ( $1.0 \text{ mg/l}^{-1}$ ) for three weeks. Well developed somatic embryos were transferred to a medium supplemented with ABA, paclobutrazol, or ancymidol, ( $0.5 - 10.0 \text{ mg/l}^{-1}$ ),  $\text{GA}_3$  ( $0.5 - 5.0 \text{ mg/l}^{-1}$ ), polyethylene glycol (PEG-4000) ( $15-100 \text{ mg/l}^{-1}$ ) and sucrose (6%) for their further development into plantlets and *in vitro* tuberization. The conversion of somatic embryos was found to be optimum in the  $\text{GA}_3$ -containing medium. Converted somatic embryos developed tubers on MS basal medium devoid of phytohormones, when incubated for two months (Figure 2F). More than 50 percent of cultures showed precocious *in vitro* flowering. Plants were transplanted to a sand:peat moss mixture and kept in growth chambers for hardening. Besides, primary embryogenesis, secondary embryogenesis was also observed (Figure 2C-E). Secondary somatic embryos emerged from the cotyledonary leaf base and tuber region of the primary somatic embryos. The method developed could be used for mass propagation of pathogen-free tubers and also has application in genetic transformation studies in *C. yanhusu*.

### **2.3. Case study 3: secondary plant metabolite production through callus (*Salvia miltiorrhiza*) and cell suspension culture (*Dioscorea doryophora*).**

#### **2.3.1. Production of cryptotanshinone from callus of *Salvia miltiorrhiza***

*Salvia* belongs to family Lamiaceae and consists of about 900 species. Some species of genus *Salvia* have been cultivated worldwide for use in folk medicine and for culinary purposes (Lu and Foo, 2002). Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge, is one of the most popular Chinese medicines used widely in improving 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; Dixon and Paiva,

1995). Its active principles tanshinones, a group of quinoid diterpenes attracted particular attention of medicinal chemists and clinicians because many of them exhibit significant antibacterial, anti-dermatophytic, antioxidant, and anti-platelet aggregation activities (Honda et al., 1998; Gao et al., 1979; Jiang et al., 1994; Cao et al., 1996; Luo et al., 1988). Also, these compounds have been used in the treatment of mastitis and wound infection. Tanshinone are wide spread in the genus *Salvia* and about 50 tanshinones so far have been identified in *S. miltiorrhiza* and other *Salvia* species. Since, Dan-shen preparations have considerable commercial value, there has been a continued interest in development of tissue culture based approaches for production of tanshinones. Plant cell cultures have a higher rate of metabolism than intact differentiated plants and can be cultured at large scale from which secondary metabolites can be extracted. The advantage of this method is that it can provide reliable quality natural products round the year on a sustainable basis.

In our group, cryptotanshinone production in *in vitro* propagated callus of *S. miltiorrhiza* induced by application of cytokinin has been reported (Wu et al., 2003). Aims of the present study were (a) to standardize an efficient protocol for induction and proliferation of callus of *S. miltiorrhiza*, (b) to analyze the content of cryptotanshinone, a quantitatively predominant tanshinone, in callus cultures and in the commercially available marketed crude drug (processed underground parts of *S. miltiorrhiza*), and (c) isolation and identification of cryptotanshinone using NMR and Mass spectral data.

**Tissue culture:** Explants used in the present study were taken from the plants grown under controlled conditions. Callus was induced on the cut surface of the leaf explants ( $5 \times 5\text{-mm}$ ) cultured on MS basal medium supplemented with 2,4-D ( $1.0 \text{ mg l}^{-1}$ ). The callus grew rapidly into a friable pale yellowish mass on this medium and was analyzed for cryptotanshinone by HPLC. The results revealed that it

contained small amounts of cryptotanshinone ( $0.26 \pm 0.05$  mg/g dry wt, Table 2). To increase the cryptotanshinone production, callus was grown on MS medium containing BA. Production of cryptotanshinone remarkably enhanced by omission of 2,4-D in the medium. The yield of cryptotanshinone varied with the concentration of BA in the MS basal medium. The callus was maintained on MS medium containing BA ( $0.2 \text{ mg l}^{-1}$ ) up to two months to ascertain the influence of incubation period on the accumulation of cryptotanshinone. Maximum yield of cryptotanshinone (4.59 mg/g dry weight) was observed in callus cultured for sixty days (Table 2). The age of the culture directly influenced the accumulation of cryptotanshinone. In *S. miltiorrhiza*, as cryptotanshinone is a phytoalexin, it is usually pro-

duced only under stressed conditions. In the present study, it is evident that, callus cultured on MS basal medium supplemented with BA ( $0.2 \text{ mg l}^{-1}$ ) could be used for *in vitro* synthesis of cryptotanshinone. Also prolonged incubation (sixty days) of the callus cultures resulted in substantial accumulation of the cryptotanshinone.

**Quantitative analysis:** For quantitative analysis, peak areas were used to calculate the amount of cryptotanshinone present in different plant material as compared to the standard. The calibration plot was linear. Cryptotanshinone content in callus grown on MS medium containing BA ( $0.2 \text{ mg l}^{-1}$ ) cultured for a period of one week to two months has been shown in Table 2.

**Table 2.** Influence of culture media and age of callus on cryptotanshinone content in callus and its comparison with market crude drug (underground parts) of *Salvia miltiorrhiza*.

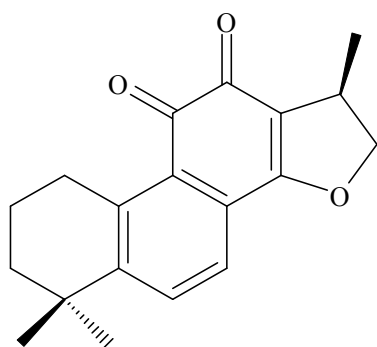
Age of callus (days)	Medium composition BA, 2,4-D ( $\text{mg l}^{-1}$ )	Cryptotanshinone (mg /g of dry wt.)
Market crude drug	--	$0.35 \pm 0.07$
8	MS + BA (0.2)	$1.78 \pm 0.02$
16	MS + BA (0.2)	$2.32 \pm 0.16$
24	MS + BA (0.2)	$3.23 \pm 0.02$
30	MS + BA (0.2)	$3.73 \pm 0.01$
60	MS + BA (0.2)	$4.59 \pm 0.09$
60	MS + BA (0.5) + 2,4-D (1.0)	$0.26 \pm 0.05$
60	MS + 2,4-D (1.0)	$0.09 \pm 0.02$
60	MS basal medium (Control)	$0.00 \pm 0.00$

-- Not applicable

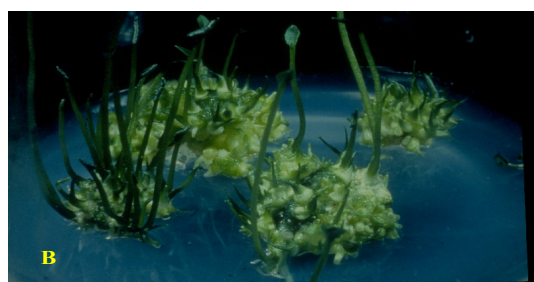
**Isolation of cryptotanshinone:** The compound was isolated from callus cultured on MS medium containing BA ( $0.2 \text{ mg l}^{-1}$ ) for a period of two months. The methanol soluble samples of freeze-dried callus were concentrated and chromatographed over silica gel column using benzene and mixtures of benzene and ethyl acetate as eluent with increasing polarity. Fractions obtained from 8% ethyl acetate were concentrated under vacuum and purified further by column chromatography

followed by preparative TLC (benzene:ethylacetate in 8:2 ratio) to obtain cryptotanshinone (15 mg). The structure of cryptotanshinone was identified by NMR and Mass spectral data and confirmed further by comparison of physical and spectral data with those of authentic samples.

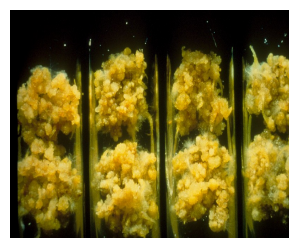
Cell suspension culture could be used for the large-scale culturing of plant cells from which secondary metabolites could be extracted (Balandrin and Klocke, 1988).



**Figure1.** Chemical structure of cryptotanshinone



**Figure 1A.** Shoot regeneration in protocorm-like bodies in *Pinellia ternata* (4 weeks after culture in MS basal, liquid medium).  
**1B:** Multiple shoot induction in protocorm-like bodies in *Pinellia ternata* (2 weeks after culture in MS basal, agar medium).  
**1C:** Shoot elongation in *Pinellia ternata* (4 weeks after culture in MS basal, agar medium).



**(a)**



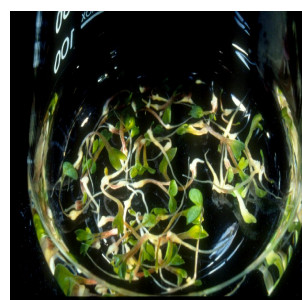
**(b)**



**(c)**



**(d)**



**(e)**



**(f)**

**Figure 2A:** Callus induction in *Corydalis yanhusuo*.  
**2B:** Shoot regeneration in callus.  
**2C:** Induction of somatic embryos from basal part of the young shoot in *Corydalis yanhusuo*.  
**2D:** Somatic embryos obtained from liquid culture medium.  
**2E:** Plantlet development from somatic embryos in *Corydalis yanhusuo*.  
**2F:** Tuber formation in plantlets derived from somatic embryos of *Corydalis yanhusuo*.

Extraction of some complex and medicinally important compounds from cell suspension culture as secondary metabolites will minimize collection from the natural habitats and thus, alleviate the pressure on wild plants.

### 2.3.2. Production of Diosgenin in cell suspension culture of *Dioscorea doryophora* Hance.

*Dioscorea* belongs to family *Dioscoreaceae*. Species are frequently used as tonic in Chinese traditional medicine. *Dioscorea doryophora* Hance, a Taiwanese species, whose tuber possesses high quality and higher quantity of active components, is being widely propagated by farmers in Taiwan (Huang et al., 1993). The tuber is in great demand as it is used both as crude drug as well as food. The most important active principle discovered in the tuber is diosgenin, which can be used as a precursor of many important medicinal steroids such as prednisolone, dexamethasone, norethisterone and metenolone (Tsukamoto et al., 1936).

For increasing yield and facilitate purification process of diosgenin, we have established a cell suspension culture of *Dioscorea doryophora* Hance (Yeh et al., 1994). Finely dispersed cell suspension cultures could be obtained by culturing both microtuber- and stem node-derived callus in liquid culture medium supplemented with 2,4-D ( $0.1 \text{ mg/l}^{-1}$ ), sucrose (3%) and incubation on a rotary shaker at 120 rpm. Although 6% sucrose was found to be optimum for the growth of cell suspension culture, however, it was observed that cells cultured in 3% sucrose containing medium produced higher level of diosgenin. Analysis by HPLC revealed that both microtuber- and stem node-derived suspension cells contained diosgenin. Content of diosgenin in various parts of plant differed significantly (Table 3). Diosgenin content, as high as 3.3% per gram dry weight was obtained in microtuber-derived cell suspension culture compared to the stem node-derived callus cell

suspension, which contained only 0.3% (Table 4). 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 the production of Diosgenin at commercial level.

**Table 3.** Diosgenin concentration of callus from different explants of *Dioscorea*

Source of callus	Diosgenin content (%)
Seed	0.4 <sup>cd *</sup>
Leaf	0.6 <sup>c</sup>
Root	1.57 <sup>b</sup>
Stem	0.0 <sup>e</sup>
Node	0.3 <sup>d</sup>
Microtubers ( <i>In vitro</i> )	3.3 <sup>a</sup>
Microtubers (Field grown)	3.2 <sup>a</sup>

\* Means in each vertical column followed by same letter are not significantly different at 5% level using MRT (Duncan's multiple range test).

**Table 4.** Diosgenin concentration in cell suspension from two different explants of *Dioscorea doryophora*

Source of cell suspension	Diosgenin content (%)
Node	0.3 <sup>b</sup>
Microtubers ( <i>In vitro</i> )	3.3 <sup>a</sup>

\* Means in each vertical column followed by same letter are not significantly different at 5% level using MRT (Duncan's multiple range test).

## 2.4. Conclusions

With the application of tools of plant biotechnology, it is now possible to produce a large number of pathogen-free medicinal

plants indigenous to Taiwan. These *in vitro* raised plants can be re-introduced in their natural habitat or cultivated at commercial level under controlled environment. Also, *in vitro* techniques can provide tools for safe exchange of germplasm across the globe. The case studies presented in the report, have clearly demonstrated that different techniques of plant biotechnology like shoot morphogenesis, embryogenesis, callus; and cell suspension cultures could be used for not only augmenting the plant population, but also for commercial production of medicinally important compounds. Thus, a goal of sustainable utilization and maintenance of medicinally important plant resources indigenous to Taiwan is very much achievable.

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