Present Status and Prospects of *in vitro* Production of Secondary Metabolites from Plants in China

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Abstract

During the past two decades, China has seen her great progress in plant biotechnology. Since the Chinese market of herb medicine is huge, while the plant resources are shrinking, particular emphasis has been placed in plant tissue and cell cultures of medicinal plants, this includes fast propagation, protoplast isolation and regeneration, cell suspension cultures and large scale fermentation. To optimize culture conditions for producing secondary compounds *in vitro*, various media, additives and elicitors have been tested. Successful examples of large scale culture for the secondary metabolite biosynthesis are quite limited : *Lithospermum erythrorhizon* and *Arnebia euchroma* for shikonin derivatives, *Panax gingseng, P. notogingseng, P. quinquefolium* for saponins, and a few other medicinal plants.

Recent development of genetic transformation systems of plant cells offered a new approach to *in vitro* production of secondary compounds. Hairy root induction and cultures, by using Ri-plasmid, have been reported from a number of medicinal plant species, such as *Artemisia annua* that produces little artemisinin in normal cultured cells, and from *Glycyrrhiza uralensis*. In the coming five years, Chinese scientists will continue their work on large scale cell cultures of a few of selected plant species, including *Taxus* spp. and *A. annua*, for the production of secondary metabolites with medicinal interests, one or two groups of scientists will be engaged in molecular cloning of the key enzymes in plant secondary metabolism.

Key words : plant cell culture; secondary metabolism; herb medicine

Introduction

Plants have a specific chemical mechanism to adapt themselves to the changing environment: the secondary metabolism, which produces the richest pool of organic compounds in the earth. Many plant secondary metabolites can be used by human beings as medicine. Taxol, an anti-cancer diterpene alkaloid from *Taxus* trees, and artemisinin, an anti-malarial sesquiterpene lactone from *Artemisia annua*, are two well known examples. In addition, many principle components of fragrance, spice, food additives and natural pigments are also plant secondary metabolites.

Asian countries, including China, have a long history of using herb medicines. With the rapidly growing population and the limited plant resources, wild plants can no longer satisfy the expending market and, more seriously, more than 60 species of medicinal plants in China have been considered endangered or nearly so, and need protection (Jia 1995). Plant tissue and cell culture techniques have opened a new way not only for the fast propagation of medicinal plants, but also made it possible to produce chemicals by cells *in vitro*. Chinese scientists have made their efforts in this area.

Tissue Culture of Medicinal Plants

Tissue culture and *in vitro* propagation

Callus induction and culture is usually the first step leading to various biotechnic and genetic manipulations of plants. In the past twenty years, about 500 plant species have been cultured *in vitro* by Chinese scientists, of these more than 20% are of medicinal interests. Some recent cultured plants for secondary metabolites include *Gentiana manshurica* (Zhang *et al.* 1992), *Gardenia jasminoides* (Zhong *et al.* 1994), *Stevia rebaudianum* (Shen 1995), *Taxus cuspidata* (Yu 1994), *T. Yunnanensis, T. wallichiana, T. chinenesis and T. baccata* (Sun *et al.* 1995).

Tissue culture offers us an invaluable substitute for traditional plant propagation: the rapid propagation *in vitro*. As regenerated plants have the

same genetic make-up as their parents, the original capability of synthesizing secondary metabolites will be maintained. By means of *in vitro* propagation, wild plant resources that are shrinking every day could be effectively protected, especially for those woody and perennial plants (Table 1).

Table 1. The endangered, rare and threatened medicinal plants that have been porpagated *in vitro*

Endangered	Rare	Threatened
Panax ginseng (Zhang & Zhang 1984)	*Eucommia ulmoides* (Zhang 1988) *Glycyrrhiza uralensis* (Li *et al.* 1986) *Gingko biloba* (Luo 1984)	*Coptis chinensis* (Ding *et al.* 1984) *Corydalis yanhusuo* (Zhang *et al.* 1979) *Fritillaria palliddiflora* (Zhong *et al.* 1984) *F. ussuriensis* (Zhou 1983) *Morinda offcinalis* (Wu *et al.* 1985) *Saposhnikovia divaricata* (Sheng & Chen 1990)

Protoplast

Plant protoplast techniques have been extensively applied in somatic hybridization, cell line screening, cellular physiology and biochemistry, and genetic manipulation. For higher plants protoplasts have been reported from more than 360 species (Xu & Xue 1995). Although these investigations have been concentrated on crops, more than 26 species of medicinal plants have been under a similar study (Table 2). Earlier reports given by Chinese scientists include isolation and plantlet regeneration of protoplast of *Solanum melogena* var. depressum (Jia & Potrykus 1981), *Datura innoxia* (Lu *et al.* 1982), *Rehmannia glutinosa* (Xu and Davey 1983), and *S. nigrum* (Wang & Xia 1983). Recently in our laboratory, Liu *et al.* (1995) compared various factors affecting *Lithospermum erythrorhizon* protoplast formation and division.

Table 2. Medicinal plant regeneration from Protoplasts (from Xu and Xue 1995)

Species	Material sources	Regeneration
Angelica dahurica	embryoid callus	EP
A. sinensis	root callus	CP
Anthriscus sylvestris	embryoid callus	EP
Asparagus officinalis	embryoid callus	EP
Astragalus grunbovis	callus	CP
A. hyangheensis	cotyledon	CP
A. melilotoides	leaf	CP
A. tennis	leaf	CP
Bupleurnm scoronerifolium	embryoid callus	EP
Cnidium monnieri	suspension cells	EP
Codonopsis pilosula	hypocotyl callus	CP
Datura innoxia	callus	CP
Digitalis lanata	leaf	CP
Heracleum moellendorffii	callus	EP
Levisticum officinale	callus	EP
Ligusticum wallichii	hypocotyl	EP
Lycium barnarum	hypocotyl	CP
Panax ginseng	embryoid	EP
Peucedanum praeruphtorum	suspension cells	EP
Petasites japonicus	leaf	CP
Pinellia ternuta	leaf	CP
Remannia glutinosa	leaf	CP
Saposhnikovia divaricata	suspension cells	EP
Solanum melogena var. *depressum*	leaf	CP
Solanum nigrum	leaf	CP

CP : plant regeneration from callus differentiated shoot; EP : plant regeneration from embryoid.

Production of Secondary Compounds

Medium and culture conditions

Medium composition is a key step not only for callus induction and cell growth, but also for regulation of secondary metabolism. Zhang *et al.* (1992) tried three basic media for *Gentiana manshurica*, and found that both MS

and B5, but not White, were suitable for callus growth and gentiopicroside formation. Phytohormone composition is probably the most important factor in medium formula. For callus culture of *Panax notoginseng*, 0.7 ppm of KT and 2.0~3.0 ppm of 2,4-D were found to be optimal (Zheng & Wang 1989). Since 2,4-D is a potential health hazard, it has been removed from many of recent experiments. For example, 1 mg of IBA and 0.23 ㎎ of KT in 1L of B5 medium were considered optimal for callus growth and pigment formation of *Gardenia jasminoides* (Zhong *et al.* 1994). IBA (5 ㎎/L) and KT (0.2 ㎎/L) were also the only regulators applied in suspension culture of *Panax quinquefolium* by Fan and Li (1993). It is interesting to note that no hormones at all were needed for high production of shikonin derivatives in cultures of *Arnebia euchroma*, a Central Asian species of *Boraginaceae*, though IBA (0.2 ㎎/L) and KT (0.5 ㎎/L) in LS medium were beneficial to callus and cell growth; thus Ye *et al.* (1992) developed a two medium system, the growth medium with IBA and KT, and the production medium without. Similar strategy was developed for callus culture and pigment formation of *G. jasminoides* (Zhong *et al.* 1994).

Various nutritional additives may have significant effect on cell growth and secondary metabolism. Coconut milk, for instance, when in 10% concentration, may increase cell growth by three times and double the saponin production of *Panax notoginseng* (Table 3), however, the cost is consequently increased (Zheng 1994). It was also reprted that 5% of coconut milk could increase the shikonin pigment concentration of the callus of *Onosma paniculatum* by five times (Zhou et al. 1992). Metal ions also play a role. Copper, for instance, when added to M-9 medium at 30 μM, increased shikonin production by 32% (Ning and Cao 1994).

Cell lines

Different cell lines may very greatly in their growth rate and target component productivity. Ye *et al.* (1992) selected four cell lines of *Arnebia euchroma* with high growth rate and pigment production : AC-3, AC-12, AC-33 and AC-34. Zheng (1989) and his colleagues treated *Anisodus acutangulus* calli with ^{60}Co rays and then selected a cell line with scopolamine content 30% higher than the control, but the productivity did not increase because of decreased growth rate. With plating clone techniques, Luo *et al.* (1994) cloned a cell line of *Panax ginseng*, PG-180, with yield of oligosacharrins twice as much as that of control. Liu *et al.*

(1995) obtained somaclones of *Lithospermum erythrorhizon* through protoplast, and the resulted cell lines showed both high growth rate and shikonin production. One of the cell lines had the pigment productivity 2.5 times higher than the parent line, and kept stable in a period of 80 days under examination.

Biotransformation and elicitation

Precursors of target secondary products, when added to culture medium, may promote biosynthesis of the compound of interests. Phenylalanine is a precursor of shikonin, supplementation with it is in *Onosma paniculatum* cell cultures increased the accumulation of shikonin derivatives (Wang, M., unpublished results). In isoprenoid pathway, Zhou *et al.* (1992) claimed that supplement with 200 ppm farnesol in cell cultures of *Panax notoginseng* increased the saponin production by 75%; however, without phosphorylation, farnesol can not be a precursor of terpenoids. Fan *et al.* (1992) reported 50% enhancement of saponin biosynthesis in *P. quinquefolium* cells after addition of squalene to the medium.

Table 3. Effects of additives on callus growth and saponin production of *Panax notoginseng* (Zheng 1994)

Additives(%)	Biomass increased	Saponins	
		content(% dw)	production(㎎)
Coconut milk(10)	89.6	5.37	7.34
Casein hydrolysate(0.2)	56.2	6.39	5.96
Pollen fragment(0.2)	34.2	4.46	3.18
Active carbon(0.2)	-2.1	4.20	1.47
Control	28.9	4.76	3.14

Start with 40 ㎎ callus/20 mL medium, cultured for 30 days.

Many plant secondary metabolites have antimicrobial activity, this possibly forms the base of medicinal interests. Those low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms are termed phytoalexins (Hans *et al.* 1994). When in cultured condition, plant cells have similar responses to phytopathogenic microorganism extracts (elicitors). Thus elicitation provides

a model for investigation of regulatory mechanism of plant secondary metabolism and an approach to enhancing target compound production. Ning *et al.* (1994) treated *Onosma paniculatum* cell cultures at growth phase with extracts of an *Aspergillus* species, and this doubled the cellular content of shikonin pigments, but the total production was increased by only 20%, because of depressed cellular viability. Fang *et al.* (1992) tested effects of extracts of six fungi on the saponin production in cell cultures of *Panax quinquefolium*. Neither of the reports showed a significantly increased component production, possibly because neither saponin nor shikonin derivatives can be considered phytoalexins and, the compound concentration in the cell before treatment was already very high. Gan *et al.* (1992) reported the enhancement of α-tocopherol production by 3.5 times when Ginseng oligosaccharides were added to the cell culture of *Carthamus tinctorius*, partly due to increased cellular growth rate. Again α-tocopherol is unlikely a phytoalexin. Recently Li *et al.* (Li, B. L., J. H. Zhao and R. Q. Cao, unpublished result) treated *Taxus yunnanensis* cell suspension cultures with crude extracts of *Botrytis* sp. at 200 ㎎ halphae/L, after 30 days the taxol content in cells and medium was increased by only 35% in comparison with control(0.02% dw). A typical example of elicitation is the biosynthesis of cotton sesquiterpenoid phytoalexins, and this will be discussed later.

Suspension and large scale cultures

Advatages of cell suspension cultures include higher cell growth rate and feasibility of varying culture conditions for secondary metabolite production. The growth rate of *Arnebia euchroma* suspension cells reached 22.0 g dw/L month and the content of shikonin derivatives was around 14% dw on 25th day post transfer (Ye *et al.* 1992).

For industrial application suspension cells may be cultured in a fermentator, the volumes vary from one to several hundred liters, and the aeration is achieved by either mechanical stirring or airlift apparatus. Some results of large scale cultures are listed in Table 4.

Collection and recovery of the principle components from liquid medium is another key step in successful large scale fermentation for plant secondary metabolites. Chen and Hou(1994) compared five different methods for recovery of shikonin from a 10 L reactor containing *Arnebia euchroma* cell cultures (Table 5). Although the recovery percentage of resin absorb is not

as high as other methods, these investigators prefer this approach because it is fast, simple and cheap. In order to avoid organical solvent extraction, Hou *et al.* (1991) applied amberlite XAD-2 resin to collect jatrorrhizine from *Berberis julinae* cell culture medium.

Table 4. Large scale cultures of plant cells for secondary metabolites

Plants	Products	Content(%)	Vol.	Bioreactor	Sources
Panax notoginseng	saponin	11.2	10L	stirring	Zuou *et al.* 1992
		9.48	4L	out-loop airlift	Hou *et al.* 1991
		2.6	4L	inner-loop airlift	Hou *et al.* 1991
P.quinquefolium	saponin	0.6	10L	stirring	Zhou *et al.* 1991
P.ginseng	saponin	5.5	10L	stirring	Zhou *et al.* 1991
Arnebia euchroma	shikonins	14.3	20L	out-loop airlift	Ye & Li 1995
		12.06	30L	airlift	Liu *et al.* 1995
Taxus yunnanensis	taxol	>0.1	10L	stirring	Gan *et al.* 1995
Glycyrrhiza uralensis	echinatin	0.18	25L	inner-loop airlift	Chen *et al* 1994

Table 5. Recovery of shikonin pigment from large scale cell cultures of *Arnebia euchroma* (Chen & Hou 1994)

Recovery methods	Pigment(mg/100 mL)		Collection percentage(%)
	recovered	unrecovered	
Filtration	112.7	13.0	89.7
Centrifugation	103.0	27.5	78.9
Extraction	131.6	/	100
Lyophilization	138.1	/	100
Resin absorb	98.7	26.0	79.1

Biochemistry and Molecular Biology

Enzymology

Isoenzymes of peroxidase of cultured plant cells have been investigated for quite a number of species, partly because of the feasibility of the technique. Zhong *et al.* (1994) found that, for *Gardenia jasminoides,* the yellowish cell line (pigment containing) and white cell line (pigment not detected) exhibited different isoperoxidase patterns. Hou and his colleagues carried out a series of experiments of *Berberis julianae* to examine the isoenzyme patterns in relation to cell growth and secondary metabolite formation (Hou *et al.* 1988a, 1988b, 1990, Chiu *et al.* 1992)

During examine the extracellular polyphenol oxidases in the liquid medium of cell suspension cultures of rosemary (*Rosmarinus officinalis*), Cai *et al.* (1993a) isolated two proteins (40 and 130 kd) with laccase activities. They found hydroxyindoles a new class of laccase substrates (Cai *et al.* 1993b). These results are of value in isolation of biologically active substances for hair coloring.

Molecular cloning of key enzymes

In cotton (+)-δ-cadinene synthase, a sesquiterpene cyclase, is a key enzyme at the first committed step leading to formation of hemigossypol and related phytoalexins. Two cDNA clones, CAD1a and CAD14a, coding for (+)-δ-cadinene synthase, were isolated from a cDNA library constructed from mRNAs of suspension cells of *Gossypium arboreum,* after elicitation with extracts of *Verticillium dahliae* (Chen *et al.* 1995). The cotton enzyme showed 40% identity at deduced peptide level to a tobacco sesqiterpene cyclase, which is also elicitor-inducible (Facchini & Chappell 1992). With CAD1a cDNA as a probe, a 10 fold enhancement of the mRNA of the cotton sesquiterpene cyclase was revealed by blotting the total RNA taken from cells at 6 hours after elicitation (Fig. 1), and the cyclase amount was increased by 6 fold within 24 hours after elicitation, according to Western blotting. The formation of sesquiterpene aldehyde, including gossypol, increased by 80 fold in 72 hours of elicitor-treatment (Heinstein 1985). The significance of this enzyme is highlighted by the fact that the

cyclic product is the most widely distributed sesquiterpene in plants. These data link the process that cell wall fragment of the fungal pathogen *V. dahliae* initiates a signal through an unknown mechanism that ultimately results in transcriptional activation of the genes encoding (+)-δ-cadinene synthase. The genetic clones are now under investigation in this laboratory.

Genetic transformation

Plant genetic engineering combines technology of DNA recombination, plant tissue and protoplast culture and genetic transformation. This has had a great impact on improvement of crop plants. However, much less work has been done on medicinal plants for secondary product formation. In China, there have been several reports on medicinal plant transformation by means of *Agrobacterium*-mediated gene transfer (Zhang 1994).

Wang *et al.* (1994) induced crown gall of *Catharanthus roseus* by infection of the callus with *A. tumefaciens*, the induced crown gall cells were then cultured in MS medium without hormones, which showed some advantages in both growth rate and alkaloid formation over callus culture.

A. rhizogenes transfers a segment of DNA containing a root-inducing fragment to the plant cell nucleus which subsequently expresses these genes. The newly developed organs can be excised and grown independently to yield hairy root cultures, show a highly heterogenous structure containing much meristematic tissue, and exhibit stable growth in medium devoid of growth regulators. Most importantly, there is no antagonism between fast growth and secondary metabolite accmulation. According to Qin *et al.* (1994a) there have been more than 95 species of medicinal plants that have been transformed by Ri plasmid. In China, the earliest investigation of hairy root induction was performed on *Solanum nigrum* (Wei *et al.* 1986). Other species of which hairy root was reported include *Scopolia japonica, Fagopyrum cymosum* (Zhang 1994), *Glycyrrhiza uralensis* (Chen *et al.* 1994), *Artemisia annua*(Qin *et al.* 1994b), *Gentiana mansurica* (Hong *et al.* 1995) and *Salvia multiorrhiza* (Zhang *et al.* 1995, Hu 1995). The accumulation of diterpenoid quinones in hairy root of *S. multiorrhiza*, in 20 days of culture, reached 43 ㎎/g dw which is 40 fold of the content in roots of 3~4 years old plants (Zhang *et al.* 1995).

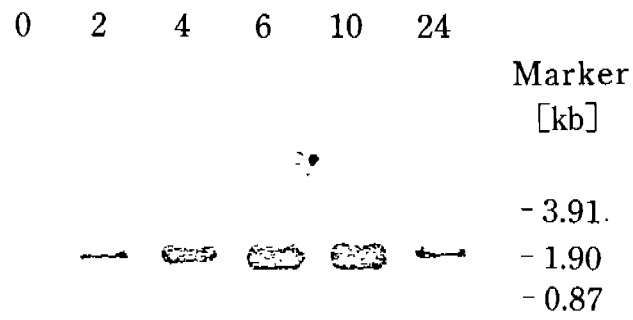

Fig. 1. Northern blot analysis of total RNA extracted from *G.arboreum* suspension cells after elicitation for 0, 2, 4, 6, 10 and 24 hours. Electrophoretically separated RNA (5 ug/lane) was hybridized with radio-labeled cDNA insert of CAD1a.

Perspective

Plant secondary metabolites are one of the major sources for the world market of flavor, fragrance, opiates and medicals. The success of *in vitro* production of these metabolites, from commercial point of view, have been limited to shikonin pigment and ginseng biomass. During past years, frankly speaking, Chinese scientists have, to a certain extent, followed Japanese and Western colleagues, although similar work was extended to systematically related species. With more interests in natural products for treatment of various virus diseases and cancers, and expending demands for plant secondary compounds, *in vitro* culture of medicinal plants and secondary metabolite production will continue to be and active area in life sciences and technology.

In next five years, on the basis of previous achievements, more work will be conducted on *in vitro* production of taxol from *Taxus* species, and artemisinin from *Artemisia annua*. The aim is clear: large scale fermentation for industrial exploitation. Hairy root culture will be a good substitute for conventional tissue and cell cultures, particularly for those plants that target

compounds are rarely accumulated in cultured cells, such as *A. annua*. Molecular cloning of key enzymes in plant secondary metabolic pathway will receive more scientific and applied interests. With some cDNA and genomic clones in hand, i. e., that of (+)-δ-cadinene synthase, molecular biologists will try to introduce the gene into plants that use this sesquiterpene intermediate to synthesize end products of interests. For agriculture, attempt to decrease accumulation of gossypol and related sesquiterpenoid toxins in cotton seeds by antisense RNA techniques is rewarding. Finally, elicitation of secondary metabolite formation in cultured plant cells may provide a model for studies of molecular mechanisms of secondary metabolism regulation and cellular signal transduction.

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