Production of Indole Alkaloids in Cell and Hairy Root Cultures of *Catharanthus roseus*

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Introduction

Catharanthus roseus produces pharmaceutically important indole alkaloids such as ajmalicine, vinblastine, and vincristine. Vinblastine and vincristine are produced by coupling two different monomeric indole alkaloids, vindoline and catharanthine (Fujita *et al.*, 1990; Endo *et al.*, 1988; Misawa *et al.*, 1988). In the plant, the former is accumulated at a relatively high level, whereas the latter is at a much lower level. However, in cultured cells usually, the former is not produced, whereas the latter is at a much lower level. Therefore, it has been considered rational to produce the dimers by coupling catharanthine obtained from cell cultures with vindoline obtained from cultivated plants (Fujita *et al.*, 1990). This paper describes a full cycle we have developed covering selection of clones and optimization of culture conditions for high yields of indole alkaloids in cell and hairy root cultures of *C. roseus*, an interchangeable system of cell suspension and hairy root cultures for maximizing biomass in the form of cells and indole alkaloids in the form of hairy roots, hairy root culture in a bubble column reactor, recovery of catharanthine using polymeric resins, isolation of vindoline from *C. roseus* leaves by supercritical fluid extraction, and production of vinblastine by chemical coupling of vindoline extracted from cultivated plants and catharanthine from hairy root cultures in *C. roseus*

Production of Catharanthine in Cell Cultures of *C. roseus*

Selection of protoclones for high yields of indole alkaloids

Two methods are generally used to select cell lines for high yields of secondary metabolites: (1) plating small cell aggregates and (2) repeatedly subculturing calli after division into smaller ones on solid medium. However, cell lines selected by these methods are not guaranteed to be of single cell origins. Alternatively, protoplast-derived clones (protoclones) may be employed to avoid chimerism of selected cell lines. Such is the case with *Lithospermum erythrorhizon* cell lines for shikonin production (Fujita *et al.*, 1984).

We have selected protoclones of *Catharanthus roseus* for high yields of catharanthine and ajmalicine (Jung *et al.*, 1992). Protoplasts were enzymatically isolated from suspension-cultured cells and cultured for 6 weeks (Fig. 1). Of the 40 isolated protoclones, protoclone VPC-10 produced catharanthine at 5.9 μg/g fresh wt of cells after 10 days of culture, although the original cell line did not produce it at a level detectable by HPLC (Table 1). Under the same conditions, protoclone VPC-15 produced ajmalicine at 133.6 μg/g, which was about 3 times the productivity of the original cell line. In addition, the indole alkaloids were qualitatively confirmed by LC-MS. The results indicate that the differences in the growth rate and indole alkaloid yield among the protoclones reflect the somaclonal variation in suspension-cultured cells. For the selection of high yielding cell lines, the methods of plating cell aggregates and repeatedly subculturing calli has been considered that they have relatively low selection efficiency. Furthermore, the productivity of isolated lines is frequently unstable. In this context, however, the selection methods of high yielding cell lines through protoplast cultures can be very effective VPC-10 line also showed a stable yield for more than one year. Although the yield of indole alkaloids of VPC-10 line was not as high as that of other reported lines (Fujita *et al.*, 1990), productivity has been improved up to 3 times by optimizing the culture conditions (Jung *et al.*, 1991).

Relationship between cell morphology and indole alkaloid production

Morphological heterogeneity of plant cells in suspension cultures greatly affects the production of secondary metabolites (Dougall 1987). For example, it was reported that production of ajmalicine, serpentine and secologanin is

unstable in suspension cultures of *C. roseus* because of heterogeneity in cell morphology (Deus-Neumann and Zenk 1984). We were interested, therefore, in determining the relationship between cell morphology and indole alkaloid production, because some of the protoclones displayed different, but stable cell aspect ratios (length/width) in long-term culture (Fig. 2) (Kim *et al.*, 1994b). As shown in Fig. 3, in the cell lines with aspect ratios below 1.5 (original cell line, VPC-22, 39, and 55) only small amounts of ajmalicine were produced and production of catharanthine was not detectable. However, in cell lines with aspect ratios over 2.8 (VPC-10, 12, and 25) the production of both compounds was significantly greater.

Production of Catharanthine in Hairy Root Cultures of *C. roseus*

Hairy root cultures have advantages over cell suspension cultures for the production of useful secondary metabolites due to their genetic and biochemical stability (Hamill *et al.*, 1987). To optimize medium conditions, the effects of the concentrations of inorganic salts in Schenk and Hildebrandt (SH) (1972) medium on catharanthine production in hairy root cultures of *C. roseus* were investigated (Jung *et al.*, 1994). The inorganic salt components could be categorized into four groups. The first group (nitrate) supported both the growth of and catharanthine production by hairy roots with incremental increases in the concentration. The second (ammonium and phosphate) yielded contradictory effects with respect to growth and production. The third (borate and molibdate) inhibited both growth and production, while the fourth (potassium indide, sulfate, and iron) did not exhibit any significant effects. Through optimization of the concentrations of inorganic salts in the medium, a two stage process of hairy root cultures with different media for growth and production was developed which enabled us to enhance the volumetric yield of catharanthine up to 60.5 mg/l (Table 2). This productivity was 5.4 times higher than that of a one stage culture in the original SH medium.

An Interchangeable System of Cell Suspension and Hairy Root Cultures of *Catharanthus roseus* for Indole Alkaloid Production

Secondary metabolite production seems to be closely linked to morphological differentiation and is generally suppressed when cells proliferate actively

(Berlin *et al.*, 1990). Thus, a sequence of a growth and a production medium, as suggested by Zenk *et al.*, (1977) or of a growth and a production phase by adding an elicitor (Eilert, 1987) was adopted to increase secondary metabolite production (Zenk *et al.*, 1977). On the other hand, in root or shoot cultures, growth and secondary metabolite production are compatible (Hirata *et al.*, 1987). Thus, indefinitely growing hairy root cultures have extended the spectrum of secondary compound production with the capacity to produce the secondary metabolites normally synthesized in the root at comparable levels (Hamill *et al.*, 1987). However, a serious problem arises from the growth characteristics of these indefinietly growing cultures. The problem is an interlaced mass of hairy roots and the necessity for inoculation or transfer of the seed culture to a mass-culture tank. Flores and Filner (1985) have mentioned that *Hyoscyamus muticus* hairy roots form calli when cultured on medium containing growth regulators, and that the callus is capable of regenerating hairy roots with a restored capacity for growth and secondary metabolite production. However, they did not realize the potential of the interchangeable cell/hairy root system in facilitating the large scale culture of hairy roots. Repunte *et al.*, (1993) have demonstrated that horseradish hairy roots reverted from cell aggregates are capable of producing peroxidase at levels comparable to initial hairy roots. They realized that the cell aggregates could be useful as "seeds" for hairy roots to avoid difficulty when inoculating and transferring a mass of intertwined hairy roots. Likewise, an interchangeable system of rhizogenic calli and hairy roots of *C. roseus* was established using the distinctive response of cells to the balance of growth regulators, i.e. the morphological interchange of roots and cell aggregates by exogenous growth regulators (Fig. 4) (Jung *et al.*, 1995a). Furthermore, the reverted hairy roots also regained productivity of the secondary metabolites of interest (Fig. 5).

Because it is more convenient to inoculate or transfer suspensions rather than hairy root cultures, the interchangeable cell/hairy root system represents a simplified process for the large scale production of hairy root cultures. Moreover, the secondary metabolite production of regenerated hairy roots from rhizogenic calli is comparable to the level of initial hairy roots. Thus, the interchangeable cell/hairy root culture system can be used for secondary metabolite production which maximizes both biomass, in the form of cells, and secondary metabolite production, in the form of hairy roots. In addition, this interchangeable system between cells and hairy roots may also be useful in the study of rhizogenesis.

Hairy Root Cultures in a Bubble Column Reactor and Recovery of Catharanthine

Acidification of medium pH below 3.0 induced the release of alkaloids from hairy roots (Jung, 1994). Degree of release was proportional to the acidification of medium, when the pH was reduced to 2.0, more than 95% of catharanthine was released from hairy roots. Polymeric resins (XAD-2, XAD-7) inhibited the growth of hairy roots, but increased the catharanthine contents about 30% higher than that without resins. Both the XAD resins did not stimulate the release of alkaloid. Even though XAD resins failed to induce the release of alkaloids from hairy roots, most of the released alkaloids were adsorbed on the resin. More than 95% of released catharanthine was recovered by XAD-2 resin. This indicates that indole alkaloids can be efficiently released from hairy roots without cell disruption and recovered by adsorption on polymeric resin. Two-liter filled hairy roots were successfully proliferated in an bubble column reactor. The inner layer of the reactor was covered with a stainless steel mesh, which enabled the hairy roots to be immobilized.

Relationship between the growth of hairy root and change of medium conductivity was determined in flask cultures (Jung *et al.*, 1995b). This relationship was used to estimate the high density growth of hairy roots in bubble column reactor during the fed-batch culture. After 45 days of culture in bubble column reactor, final concentration of hairy roots and catharanthine content were increased up to 46.5 (g dry wt/l) and 2.4 (mg/l dry wt), respectively, and the volumetric yield of catharanthine was 111.6 mg/l. Biomass of hairy roots calculated from the relationship was 44.53 g/l, which shows a deviation of 5% from the actual value of the biomass density.

Isolation of Vindoline from *C. roseus* leaves by Supercritical Fluid Extraction

Vindoline was extracted from the leaves of *C. roseus* over the ranges of 35-70℃ and 100-300 bar using supercritical carbon dioxide with and without the addition of 3 wt % ethanol as a cosolvent (Song *et al.*, 1992). The vindoline contents in the extracts were determined by HPLC and identified by LC/MS. The remarkable highest vindoline concentration, 58

wt %, was obtained at the lowest temperature, 35℃, and the highest pressure, 300 bar, of this study. The use of a cosolvent only slightly improved the extraction yields or selectivities at some experimental conditions.

Production of Vinblastine by Chemical Coupling of Vindoline Extracted from Cultivated Plants and Catharanthine from Hairy Root Cultures in *C. roseus*

Vinblastine, and anticancer agent was produced by chemical coupling of two different monomeric indole alkaloids, vindoline and catharanthine in the presence of ferric ion (Kwak *et al.*, 1993). Vindoline was efficiently extracted from the leaves of vinca (*Catharanthus roseus*) by using supercritical carbon dioxide, whereas catharanthine was chemically extracted from the *in vitro* cultured hairy roots. The extracted crude monomeric precursors were purified by a two-step preparative TLC. The coupling reaction was carried out in the 0.1 M glycine buffer (pH 2.0, 5 ml) containing 40 mM $FeCl_3$ with purified vindoline (0.3 mg) and catharanthine (0.3 mg) at 4℃. The production yields (weight %) of vinblastine and 3′, 4′- anhydrovinblastine in the products were 23.2 and 26.0, respectively. The produced vinblastine was confirmed by FAB-MS.

Conclusions and Future Perspectives

Vinblastine, vincristine and other indole alkaloids have become "old-fashioned" drugs. Their market size is now not so big enough that their commercial production cannot be considered by using the plant cell culture system. However, the production of indole alkaloids in cell cultures of *C. roseus* has served as an excellent model system for secondary metabolite production in plant cell cultures in many laboratories for over 25 years. We have developed a system covering a full process from developing cell lines to coupling of catharanthine and vindoline to produce vinblastine. In this system, we have demonstrated a unique interchangeable system of cell suspension and hairy root cultures to maximize biomass in the form of cells and secondary metabolites in the form of hairy roots and a system for isolation of vindoline from leaves by supercritical fluid extraction.

On the other hand, Yun *et al.* (1992) succeeded in enhancing the productivity of scopolamine, a tropane alkaloid, in *Atropa belladonna* by introducing cDNA clone of the gene of hyosycyamine-6β-hydroxylase, a rate-limiting enzyme in the process of catalyzing hyoscyamine to scopolamine, from *Hyoscyamus niger* using Ti-plasmid-mediated transformation. Their results suggest that the productivity of vinblastine or vincristine in *C. roseus* plants may be elevated by manipulating the expression level of the gene for a rate-limiting enzyme, if any, in the biosynthetic process of catharanthine. In this way, we may detour the coupling process for the production of dimer alkaloids and cell cultures by extracting the compounds directly from cultivated plants in the field. To do so, a plant regeneration system for this species is prerequisite. We have succeeded to establish a system for high frequency plant regeneration from anther-derived cell suspension cultures via somatic embryogenesis in *C. roseus* (Kim *et al.*, 1994a).

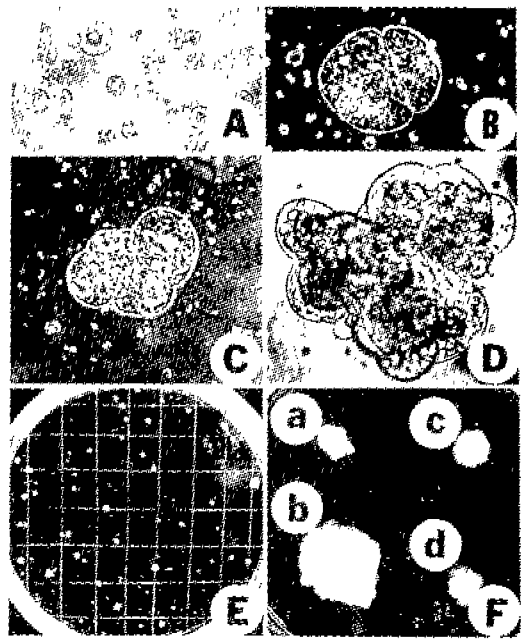

Fig. 1. Callus formation from suspension culturederived protoplasts of *Catharanthus roseus*.

A. Freshly isolated protoplast (x 100)
B. First cell division of a protoplast (x 100)
C. Second division of protoplast (x 100)
D. Colony formation from a protoplast (x 400)
E. Microcalli formation on agarose culture medium
F. Protoclones subcultured on SH agar medium
(a, VPC-5; b, VPC-6; c, VPC-7; d, VPC-8)

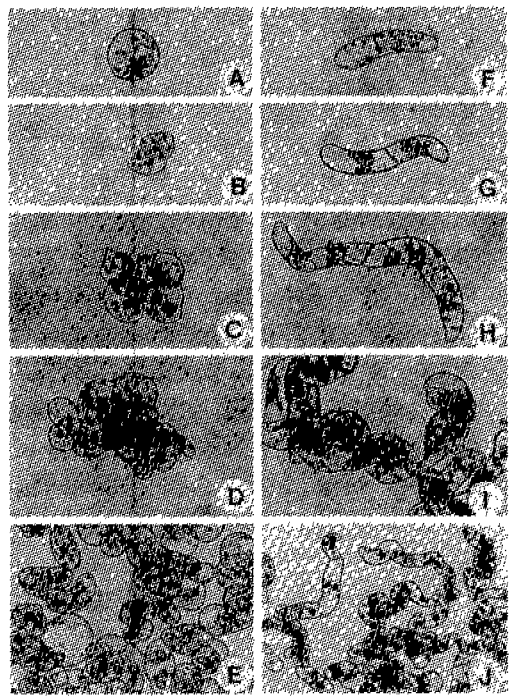

Fig. 2. Cell division and colony formation from isolated single cells of the original cell line (A to D) and VPC-10 (F to I), and suspension-cultures of the original cell line (E) and VPC-10 (J), respectively.

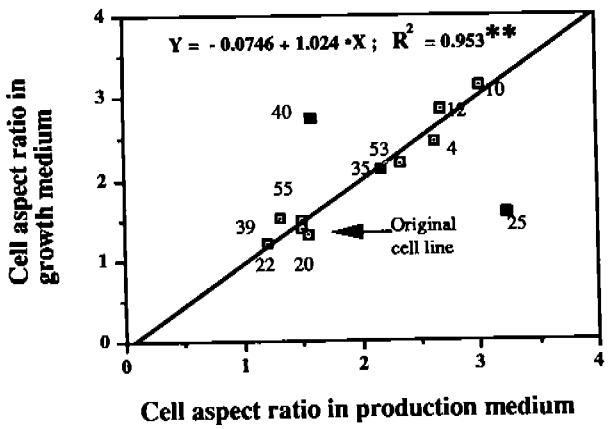

Fig. 3. The relationship between the cell aspect ratio of *C. roseus* protoclones cultured in growth medium and that in production medium. The numbers indicate VPC protoclones. When the equation of the linear regression is calculated, the cell aspect ratios of VPC-25 and 40 (■) are not included. ** Significant at P = 0.01.

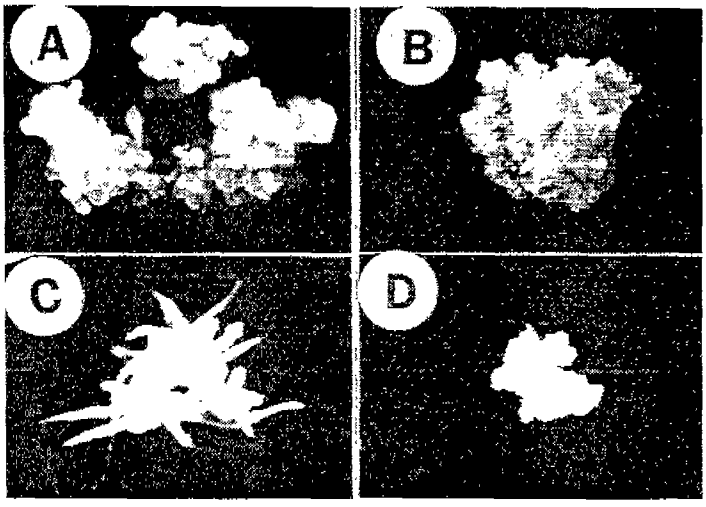

Fig. 4. Morphology changes of cell lines by growth regulators. A, Rhizogenic callus on SHNK medium; B, Nonrhizogenic callus on SHNK medium; C, Hairy roots regenerated from rhizogenic callus on 1/3 SH basal medium; D, Nonrhizogenic callus on 1/3 SH basal medium.

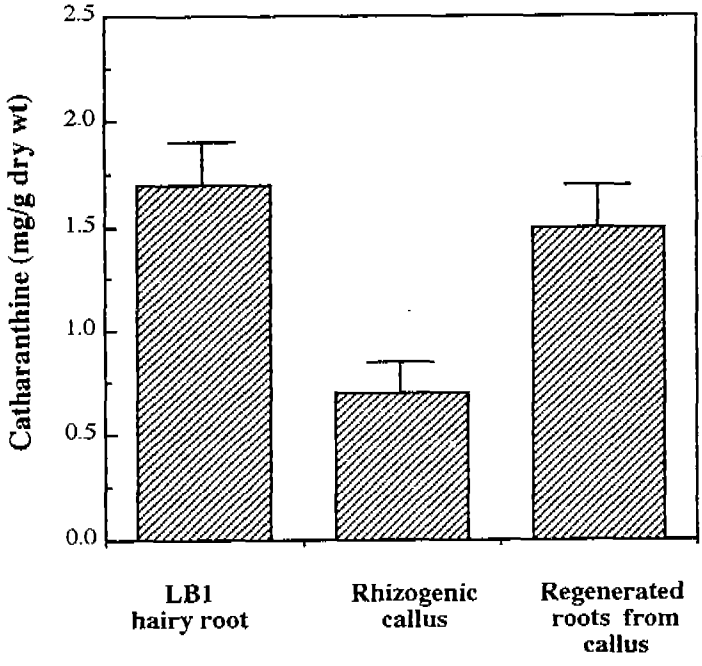

Fig. 5. Effects of morphological changes on catharanthine production in hairy root cultures. The vertical bars indicate s.e.

Table 1. Production of indole alkaloids in cell suspension cultures of selected protoclones of *Catharanthus roseus*.

Protoclone VPC-	Fresh weight of cells (g/15 ml medium)	Ajmalicine (μg/g fresh weight of cells)	Catharanthine (μg/g fresh weight of cells)
6	2.9	64.9	3.9
10	1.9	12.5	5.9
15	2.1	133.6	0.0
The original cell line	2.3	42.2	Trace*

* Trace indicates level undetectable by HPLC.

Table 2. Production of catharanthine in hairy root cultures of *C. roseus* by optimization of inorganic salt levels.

Culture condition	Biomass (g/l)	Catharanthine content (mg/g dry wt)	Culture time (d)	Volumetric yield (mg/l)	Relative productivity[a]
One stage culture					
SH	8.5	0.75	20	6.38	1.0
1/3 SH	9.5	1.8	20	16.63	2.7
GM	12.5	1.5	20	18.75	2.9
PM	7.5	3.4	20	25.5	4.0
Two stage culture					
GM→PM	19.5	3.1	35	60.5	5.4
1/3 SH→1/3 SH	19.7	1.9	35	37.4	3.4

1/3 SH, Medium containing one-third diluted inorganic salts of SH.

GM, PM, Media revised for growth and production, respectively.

[a] Relative productivity was calculated by comparing the volumetric productivity (mg/l/d) for each process to that of SH.

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