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SELECTION OF PROTOCLONES FOR HIGH YIELDS OF INDOLE ALKALOIDS FROM SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS* AND THE QUALITATIVE ANALYSIS OF THE COMPOUNDS BY LC-MS

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SUMMARY ; To produce economically important indole alkaloids by cell culture, we have selected protoclones of *Catharanthus roseus* for high yields of catharanthine and ajmalicine. Protoplasts were enzymatically isolated from suspension-cultured cells. 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. 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.

INTRODUCTION

Vinca (*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 considerable 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).

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). In this communication, we describe the selection of protoclones from suspension-cultured cells of vinca for high yields of ajmalicine and catharanthine, and the conditions of qualitative analysis of the compounds by liquid chromatography-mass spectrometry (LC-MS).

MATERIALS AND METHODS

Plant material and callus induction. To induce callus, hypocotyl segments of seedling of vinca (*C. roseus* (L.) G. Don ; cv. Little Delicata) were placed on Schenk and Hildebrandt (SH)(1972) medium supplemented with 0.5 mg 2,4-dichlorophenoxyacetic acid /l, 0.1 mg kinetin /l, and 2.0 mg 4-chlorophenoxyacetic acid /l (callus induction medium). Unless mentioned otherwise, all culture were maintained in the dark at 25 °C throughout the experiments. After 4 weeks of culture, friable, yellowish callus arose from the entire surface of the explants.

Culture of protoplasts and protoclones. After 6 weeks of culture, induced callus was transferred into SH liquid medium and cultured on a gyratory shaker (100 rpm). The cells were subcultured every ten days. Protoplasts were enzymatically isolated from suspension-cultured cells according to the method of Frearson *et al.* (1973). Isolated protoplasts were plated at a density of 1×10^5 protoplasts/ml in MS basal liquid medium supplemented with 9% mannitol, 2 mg 1-naphthaleneacetic acid /l, 0.5 mg 6-benzyladenine /l. The plating efficiency (%) was calculated after 6 weeks culture as follows : number of visible colonies divided by total numbers of plated protoplasts. Subsequently, the visible colonies (protoclones) were transferred onto the callus induction medium. After 10 weeks, each of the protoclones was separately transferred into SH liquid medium and subcultured 3-5 times before the indole alkaloid yield was measured. 0.5 g (fresh weight : fr wt) of subcultured cells were inoculated into a 100 ml Erlenmeyer flask with 15 ml of one-half strength of Murashige and Skoog's (MS)(1962) medium containing 1.13 mg 6-benzyladenine /l, 0.18 mg 3-indoleacetic acid /l, and 50 g sucrose /l (production medium). Cells were harvested by filtration after 10 days of cultures.

Extraction of crude alkaloids. The extraction of crude alkaloids was performed by a modified method of Renaudin (1984). For each sample, 1.0 g (fr wt) of cells was extracted three times with 10 ml methanol for 30 min in an ultrasonic bath (50 °C). The residues obtained by removal of methanol *in vacuo* were extracted with 1M HCl and ethyl acetate (1/1, v/v). Acidic solution was adjusted to pH 10 with 5 M NaOH and extracted 3 times with ethyl acetate. The combined ethyl acetate phase was then evaporated under reduced pressure to give the crude alkaloid extract.

Quantitative analysis of alkaloid contents. The crude alkaloid extract was redissolved in methanol and filtered through a 0.5 µm FH-type Millipore filter. This sample was loaded onto a reversed phase column, µ-Bondapack C18 column (3.9 x 30 cm). The solvent mixture of methanol, acetonitrile, and 5 mM diammonium hydrogen phosphate (pH 7.3, 3/4/3 by vol.) was eluted at a flow rate of 1ml/min, and indole alkaloids containing fractions were detected at 298 nm. The quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic alkaloids.

Qualitative analysis by LC-MS. Ajmalicine and catharanthine produced from protoclone coded VPC-10 were determined by LC-MS : Hewlett-Packard 5988A mass spectrometer equipped with a particle-beam LC-MS interface. The HPLC conditions for LC-MS were slightly adjusted for mass spectrometry as follows : the ammonium acetate was substituted for diammonium hydrogen phosphate in the mixture solvent to increase vaporization, and the solvent was eluted at a flow rate of 0.5 ml/min. The other conditions were the same as described in the above quantitative analysis by HPLC. Electron impact ionization, 300 °C ion source temperature, 70 eV and 278 mA , was used for the mass spectrometry. For the final confirmation of indole alkaloids produced by VPC-10, the crude alkaloid extracts were loaded on the LC-MS.

RESULTS AND DISCUSSION

The first cell division of protoplasts isolated from suspension-cultured cells was observed after 3 days of culture, and 40 colonies (protoclones) were formed after 6 weeks of culture (Fig. 1). The plating efficiency was 0.01 %. Of the 40 isolated protoclones, only half produced indole alkaloids such as ajmalicine and catharanthine at levels detectable by HPLC. A cell line coded VPC-6 showed a relatively high growth rate and yield of indole alkaloids. Protoclone VPC-10 produced catharanthine at 5.9 µg/g fr wt of cells after 10 days of culture, whereas the original cell line did not produce it at a level

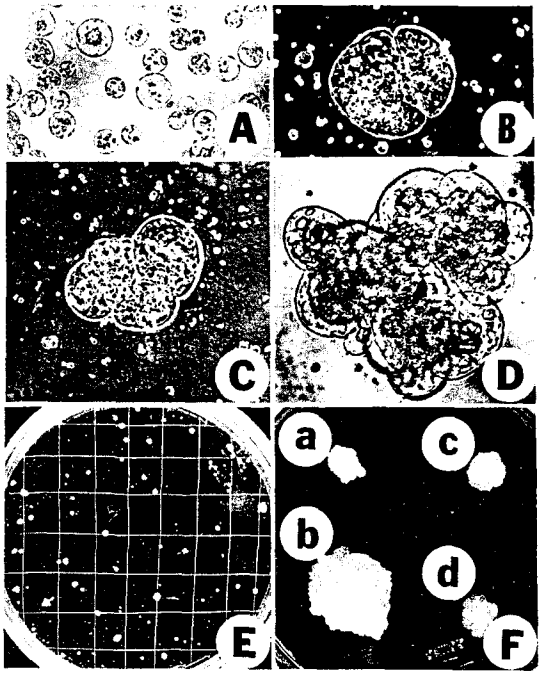

Figure 1. Callus formation from suspension culture-derived protoplasts of *Catharanthus roseus*.
A, Freshly isolated protoplasts (x 100).
B, First cell division of a protoplast (x 100).
C, Second division of a protoplast (x 100).
D, Colony formation from a protoplast (x 400).
E, Microcalli formation on agarose culture medium.
F, Protoclones subcultured on SH agar medium (a, VPC-5 ; b, VPC-6 ; c, VPC-7 ; d, VPC-8).

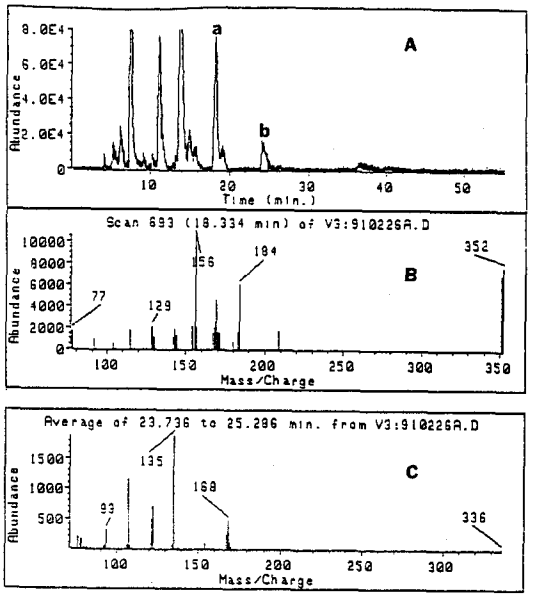

Figure 2. LC - MS analysis of ajmalicine and catharanthine produced by protoclone VPC-10.
A, Total ion chromatogram of crude alkaloids (a and b denotes the corresponding peaks of ajmalicine and catharanthine, respectively).
B, Mass spectrum of ajmalicine .
C, Mass spectrum of catharanthine.

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.

detectable by HPLC. Protoclone VPC-15 produced ajmalicine at 133.6 µg/g, which is about 3 times the productivity of the original cell line (Table 1).

The total ion chromatogram of sample indicated several peaks as shown in Fig. 2-A. Among them, two compounds detected at 18.3 and 24.4 min were putatively identified as ajmalicine and catharanthine, respectively, when their retention times were compared with those of the authentic samples. The mass

spectra of ajmalicine and catharanthine produced by VPC-10 were the same as those for the authentic samples : ajmalicine, m/z=352 (M^+, 70%), 351 (63%), 184 (55%), 169 (41%), 156 (100%), and 129 (20%) as base fragments ; catharanthine, m/z=336 (M^+, 3%), 168 (26%), 135 (100%), and 122 (37%). The identification of the compounds for the other peaks is under investigation.

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, as shown in shikonin production (Fujita *et al.*, 1984). Constabel *et al.* (1981) reported the variation in the alkaloid yields of protoclones derived from one single leaf of vinca. Apart from these, there has been no reports on the selection of protoclones for high yields of indole alkaloids. VPC-10 line also showed a stable yield for more than one year. Although the yield of indole alkaloids of VPC-10 line is not as high as that of other reported lines (Fujita *et al.*, 1990), in preliminary experiments productivity was improved up to 3 times by optimizing the culture conditions. We are now trying to select subclones from selected protoclones to improve the catharanthine yield even more.

Previously, indole alkaloids produced in plants have been identified by gas chromatography (GC) and/or mass spectrometry (MS). However, because the samples must be vaporizable and/or purified, the methods are not generally applicable. As demonstrated in this experiment, however, nearly all the samples analysable by HPLC are applicable to LC-MS without tedious vaporization and/or purification of products. Thus, LC-MS analysis is very useful method for identifying secondary metabolites : the products could be simply confirmed by retention times and mass spectra for known compounds. Furthermore, much information on the chemical structure could also be obtained from molecular and base peak ions, even for unknown compounds. we expect that the research of secondary metabolite production by plant cell cultures will take advantage of LC-MS in the coming years.

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