

Relationship between cell morphology and indole alkaloid production in suspension cultures of *Catharanthus roseus*

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Abstract. The relationship between the morphology and indole alkaloid production of *Catharanthus roseus* cells was investigated. Eleven cell lines were randomly selected from protoplast-derived clones. In each line, most of the cells maintained only one of the two shapes, either spherical or cylindrical. The cell aspect ratio (cell length/width) for most isolates was stable for more than two years of subculture. Cell division patterns of spherical and cylindrical cell isolates were different and patterns of division remained stable in each phenotype and were not considerably affected by auxin or cytokinin levels in the culture media. These observations indicate that cell morphology of our isolates is stable and probably internally determined. Production of the indole alkaloids, ajmalicine and catharanthine was significantly greater when the cell aspect ratio was more than 2.8.

Key words: *Catharanthus roseus* — Cell aspect ratio — Cell morphology — Indole alkaloids — Protoclones — Vinca

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; CPA, p-chlorophenoxyacetic acid; IAA, indole-3-acetic acid; MS, Murashige and Skoog (1962) medium; SH, Schenk and Hildebrandt (1972) medium

Introduction

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 *Catharanthus roseus*

because of heterogeneity in cell morphology (Deus-Neumann and Zenk 1984). In a previous report we described that cell heterogeneity is reduced when cultures are established from protoplast-derived cell lines (protoclones) of *C. roseus* (Jung et al. 1992). In red beet callus culture, the cell morphology is indicative of the kinds of betalain pigments accumulated in the cell (Girod and Zryd 1991). 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. In this report we compare metabolite production among *C. roseus* protoclone isolates that displayed various cell aspect ratios to the original unselected cell line.

Materials and methods

Plant material. An unselected cell line (= original cell line) derived from hypocotyl segments of vinca (*Catharanthus roseus* (L.) G. Don (cv. Little Delicata) and its randomly selected eleven protoclones (Jung et al. 1992) were maintained in liquid growth medium (see below). The original cell line contained a mixture of cell shapes, although spherical cells were predominant. However, in each of the protoclones, designated VPC-4, 10, 12, 20, 22, 25, 35, 39, 40, 53, and 55 (Jung et al. 1992), most of the cells retained only one of the two shapes, either spherical or cylindrical. Protoclones were of their single cell origin.

Cell growth and metabolite production media. Cells from each cell line were grown and subcultured in SH (Schenk and Hildebrandt 1972) liquid medium containing 2 mg l⁻¹ CPA, 0.5 mg l⁻¹ 2,4-D, 0.1 mg l⁻¹ kinetin, and 30 g l⁻¹ sucrose (growth medium) at 7 d intervals. Cells were transferred into one half-strength MS (Murashige and Skoog 1962) liquid medium containing 1.13 mg l⁻¹ BA, 0.18 mg l⁻¹ IAA, and 50 g l⁻¹ sucrose (production medium) for metabolite production. Each passage of cells to production medium was conducted by transfer of one g of fresh weight of suspension-cultured cells into 50 ml of fresh medium in 300 ml Erlenmeyer

flasks. All of the suspension cultures were incubated at 25°C in the dark on a gyratory shaker (100 rpm).

Measurement of cell growth. Fresh and dry weight of the original cell line and its protoclone VPC-10 cultured in growth medium were measured as parameters of cell growth over a culture period. Fresh weight was measured after harvesting the cells by filtration through filter paper under reduced pressure. Dry weight was measured after desiccation at 60°C for 24 h.

Examination of cell division and morphological characteristics. To observe the cell division patterns during culture, cells in the log phase (7 d after inoculation) were collected and resuspended in fresh growth medium. The suspension was then mixed with an equal amount of the same medium containing 1.2% melted agarose kept at 45°C. One ml each of the mixture was quickly poured into each well of a Nunc Petri dish (60 x 15 mm) before solidifying. After incubation in the dark, cells were periodically observed with an inverted microscope.

Measurement of cell aspect ratio. Cell aspect ratio (cell length/width) was examined in the original cell line and each of the protoclone isolates. One hundred cells of each line were randomly selected and photographed. The length and width of each cell was measured from the photographs, and the average cell aspect ratio of the one hundred cells of each line was determined. To determine whether the cell aspect ratio of each line was changed in different culture media, the aspect ratio of cells in the log phase cultured in growth medium was compared with that in production medium after 13 d of inoculation.

Analysis of indole alkaloid productivity. The original cell line and the protoclones were harvested after 13 d of culture in production medium and were subjected to solvent extraction followed by quantitative analysis of indole alkaloids using HPLC equipped with ODS column (Renaudin 1984, Jung et al. 1992). The qualitative analysis of ajmalicine and catharanthine was carried out by LC-MS (Jung et al. 1992).

Results

The growth rates, cell division patterns and morphological characteristics of two isolates (original unselected and VPC-10) were determined after transfer to growth medium. The growth curves derived from both fresh and dry weight measurements were sigmoid and growth rates were similar for each of the isolates (Fig. 1). Growth rates of the two cell lines in metabolite production medium were only about half of those in growth medium (data not shown). Changes in cell division patterns during the culture of the original cell line and VPC-10 are shown in Fig. 2. In the original cell line, the first cell division took place transversely and the next division usually perpendicularly to the plane of the first division, which resulted in the formation of small clustered colonies consisting of spherical cells. In contrast, in the VPC-10 cultures, the second and subsequent division occurred in the same plane and resulted in the

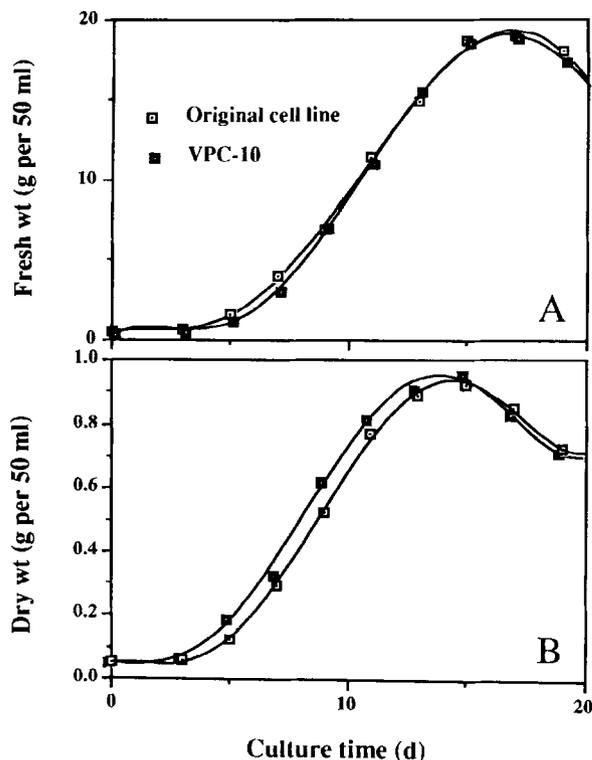


Fig. 1. Time-course of cell growth in fresh (A) and dry weight (B). The original cell line (□-□) and VPC-10 (■-■) of *C. roseus* were cultured in growth medium.

formation of colonies of cylindrical cells. Those colonies were chains of several elongated cells with septa oriented perpendicularly to the long axes of the cells. The morphological characteristics of the two cell lines were clearly distinguishable from each other and the morphological characteristics of each were maintained for over two years of subculture (data not shown).

The cell aspect ratios of all protoclone cell lines in both growth and production media were compared (Fig. 3). All isolates displayed nearly identical aspect ratios in both media, except for VPC-25 and 40. The aspect ratio of VPC-25 was noticeably increased, whereas that of VPC-40 was decreased by transferring from growth medium to production medium.

The relationship between cell aspect ratio and catharanthine and ajmalicine production for each cell line was determined (Fig. 4). 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.

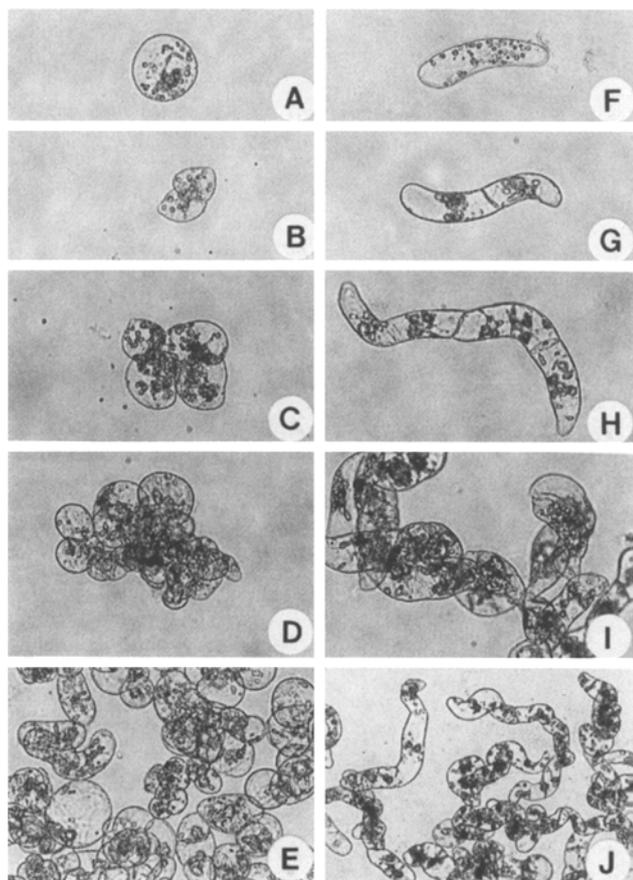


Fig. 2. Cell division and colony formation from isolated single cells of the original cell line (A to D) and VPC-10 (F to I), and suspension-cultures of the original cell line (E) and VPC-10 (J), respectively.

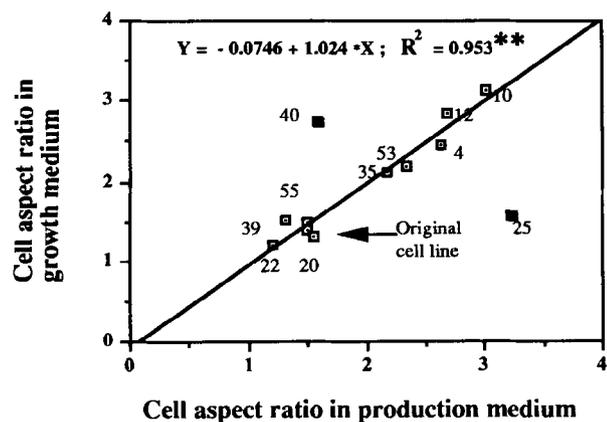


Fig. 3. The relationship between the cell aspect ratio of *C. roseus* protoclones cultured in growth medium and that in production medium. The numbers indicate VPC protoclones. When the equation of the linear regression is calculated, the cell aspect ratios of VPC-25 and 40 (■) are not included.

** Significant at $P = 0.01$.

Discussion

It was reported that the indole alkaloid production in *C. roseus* cell suspension cultures was unstable, and that the instability was due to heterogeneity in cell morphology in suspension cultures (Deus-Neumann and Zenk 1984, Dougall 1987). As shown in this study, the heterogeneity of cell morphology may be overcome by selection of protoclones which maintain a stable cell aspect ratio over a prolonged subculture. The cell aspect ratio of most protoclones was not considerably affected by auxin/cytokinin ratios in either growth or production medium (Fig. 3) or by sequential passage over two years. These observations suggest that the cell shapes are not interchangeable during cell growth, and that cell aspect ratio appears to be internally determined at the time of selection. Suzuki et al. (1990) also noted that spherical and cylindrical cells in cell suspension cultures of *C. roseus* retained a stable morphology for 7 years. Thus, we hypothesize that cell shape of each cell line is a mitotically stable characteristic and that morphology is regulated by a stabilized gene expression governing the orientation of the plane of cell division.

In this study, the cell lines (VPC-25 and 40) of which cell aspect ratios were changeable in different culture media seemed to be at the competent stage with respect to their ability to change the cell aspect ratio when exposed to appropriate exogenous growth regulators, whereas the other cell lines with highly fixed cell aspect ratios were at the determined stage. In other words, cells that are not fully differentiated are "plastic" in shape under the influence of exogenous growth regulators and cells fully differentiated are not changeable in shape unless they begin to be dedifferentiated. Girod and Zryd (1991) observed that the cell morphology corresponding to each colored phenotype, reflecting the kinds of betalains accumulated in the cell, of red beet callus is changed from one colored state into another as the growth regulators in the medium is changed. Such cell seems to be the case of the competent state.

It is intriguing that indole alkaloid production of the cell lines dramatically increased when the aspect ratio was over a critical value of 2.8 (Fig. 4). This observation indicates that there is a threshold of cell aspect ratio relevant to the indole alkaloid productivity. Suzuki et al. (1990) reported that the major components of spherical cell wall in cell suspension cultures of *C. roseus*

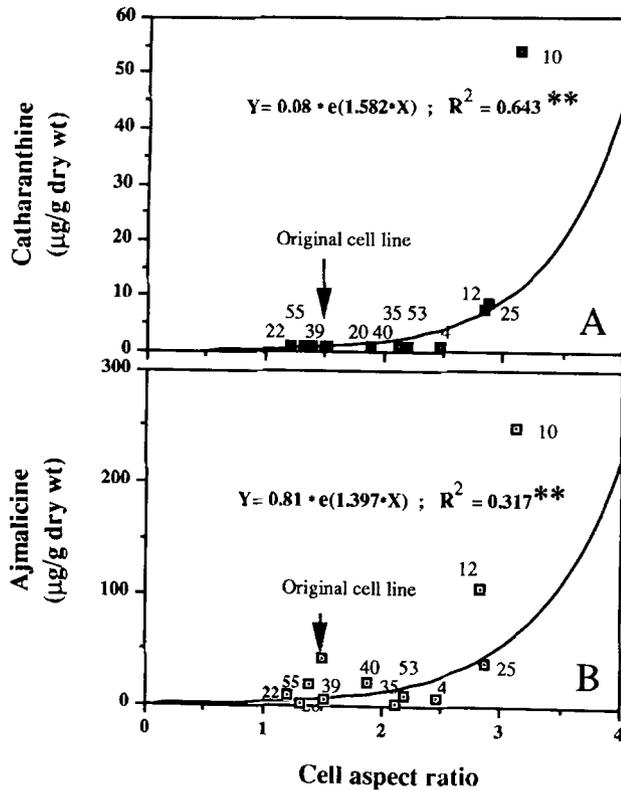


Fig. 4. The relationship between the cell aspect ratio in production medium and the productivity of catharanthine (A) ajmalicine (B) in of *C. roseus* protoclones. The numbers indicate VPC protoclones. When the equation of productivity is calculated, 1 is added to each datum to transform to the exponential curve.

* ** Significant at $P = 0.05$ or 0.01 , respectively.

are galacturonan, arabinogalactan, and xyloglucan, whereas the major component of cylindrical cell wall is arabinan, and that differences in the cell morphology might be related to differences in wall composition. Thus, we suggest that a cylindrical cell wall component such as arabinan, when secreted into culture medium from autolyzed cells, might act as an elicitor for

indole alkaloid production. It is noteworthy that in our preliminary experiments the autolysis of old cells was prominent in the stationary phase where the productivity of indole alkaloids was maximized. We will carry out experiments to determine whether the cylindrical cell wall fragments may play the role of an elicitor for indole alkaloid production.

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