Growth and alkaloid synthesis in cell lines of *Catharanthus roseus* obtained through immobilization of cells and protoplasts

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Abstract. Cells and protoplasts of *Catharanthus roseus* were immobilized with sodium alginate, agar and agarose. Cells proliferating from the matrix were established separately in liquid suspension and 5 cell lines were isolated which showed differences in their growth and alkaloid synthetic pattern. Cell lines obtained through immobilization of protoplasts yielded higher levels of alkaloids.

Keywords. *Catharanthus roseus*; immobilization; cells, protoplasts, ajmalicine.

1. Introduction

Plant cells and protoplasts have been immobilized in different matrices for investigations on secondary product biosynthesis. Under immobilized state several factors such as matrix pressure, oxygen tension, nutrient permeability, cellular exudates and cell to cell contact are known to influence the metabolic events of the cells resulting in variations of product biosynthesis (Brodelius and Nilson 1980). The immobilized cells are known to divide after prolonged period of subculture and give rise to fine suspensions comprising single cells and smaller cell aggregates (Fowler 1983). In the previous communication (Bapat et al 1986) we have reported the protocol for immobilization of *Catharanthus* cells and protoplasts in different matrices. The present report concerns the studies on growth and product biosynthesis of the cells emerging through immobilization matrices.

2. Materials and methods

Cells and protoplasts were immobilized in alginate, agar and agarose as described previously (Bapat et al 1986). For alginate immobilization cells and protoplasts were mixed with 2.5% sodium alginate (Sigma) and the mixture was pipetted dropwise into 50 ml of culture medium containing CaCl$_2$ 2H$_2$O (1.036 g/150 ml). For protoplast immobilization the osmoticum of the solution was maintained by adding 4% sucrose. The cells and protoplasts were immobilized by entrapment with molten agar or agarose medium below 30°C. After cooling the agar and agarose matrix were cut into cubes of 1 cm. The resulting alginate beads and cubes of agar and agarose were cultured in MS (Murashige and Skoog, 1962) liquid medium + 2,4-dichlorophenoxyacetic acid (1 mg/l). The cells emerging from different matrices were isolated and established on the same medium over 6 serial subcultures of 21 days. By this method 5 cell lines (i) cells immobilized in alginate, (ii) cells immobilized in agar, (iii) cells immobilized in agarose, (iv) protoplasts immobilized in agar and (v) protoplasts immobilized in agarose were established.
Growth was measured in terms of fresh weight, dry weight and packed cell volume. Packed cell volume was measured by allowing 100 ml of suspension to settle down for 20 min. The sugar content was measured on a refractometer using standard sucrose curve. For alkaloid production, cells from growth medium were transferred to production medium of Zenk et al. (1977) using 20% inoculum and grown for 30 days.

For alkaloid analysis the tissues from growth and production medium were lyophilized, powdered and extracted by the method described previously (Benjamin et al. 1990). Thin-layer chromatography of the basic extracts was carried out from silica gel plates using ethylacetate-methanol (96:4). The alkaloids were visualised by spraying with cericammonium sulphate and heating the plates at 110°C for 5 min. High performance liquid chromatography (HPLC) was carried out on Waters Associate Model (ALC/GPC 244) equipped with μ-bondapak C-18 column using solvent system methanol-diammonium hydrogen phosphate (70:30). Quantification of ajmalicine and serpentine was done using standard curves obtained from known concentrations of authentic samples.

3. Results and discussion

Cells immobilized in alginate beads proliferated and liberated cells into the surrounding liquid medium within 8–10 days, whereas cells entrapped in agar and agarose liberated cells into the liquid medium in 15–20 days. Active growth of the released cells occurred after 25–30 days. All the cell lines were fine suspensions comprising mainly single cell and aggregates of 10–20 cells. The cells were generally elongated and were of different sizes with prominent nuclei and dense cytoplasm (figure 1A).

Cell lines established from alginate immobilization showed an initial lag phase during 0–4 days followed by uniform growth up to 20 days and then reached a stationary phase as evidenced by fresh and dry weights. The packed cell volume reached 90% after 20 days. Most of the sucrose was utilized at the end of the eighth day (figure 1B). The cell lines derived from agar and agarose immobilization showed similar growth pattern during the incubation period of 20 days.

The parent cell line of *Catharanthus* produced trace amounts of alkaloids in MS liquid medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l). However, in Zenk's production medium the cells produced significantly higher levels of alkaloids of which ajmalicine was the major component. Cell lines initiated through alginate, agar and agarose immobilization of cells produced consistently lower levels of ajmalicine in Zenk's production medium as compared to the parent cell line (table 1).

Protoplasts immobilized in alginate failed to divide. However, protoplasts entrapped in agar or agarose divided and liberated cells into the surrounding liquid medium at the end of 8–10 weeks. The colonies comprised single cells and aggregates of 10–20 cells. Morphologically these cell lines were similar to those derived from cell immobilization. The cell line initiated from the protoplast immobilization showed uniform growth from the 4th day and reached stationary phase on the 12th day as evidenced by dry weight and packed cell volume increment. HPLC analysis of the basic extract of the cell lines derived through
immobilization of protoplasts showed that ajmalicine content was more in these cells. Small amounts of serpentine was also present in these extracts (table 1).

The 5 cell lines of Catharanthus in the present study derived through immobilization of cells as well as the protoplasts exhibited marked changes in their biosynthetic activities which could be attributed to the effects of immobilization and protoplasts isolation. Cell lines derived through immobilization of cells in alginate,
Table 1. Alkaloid content of cell lines derived from immobilization of cells and protoplasts of *C. roseus*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells</th>
<th>Protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ajmalicine (mg/100 g/dw)</td>
<td>Serpentine (mg/100 g/dw)</td>
</tr>
<tr>
<td>Control</td>
<td>123</td>
<td>—</td>
</tr>
<tr>
<td>Alginate</td>
<td>52</td>
<td>1.3</td>
</tr>
<tr>
<td>Agar</td>
<td>66</td>
<td>—</td>
</tr>
<tr>
<td>Agarose</td>
<td>30</td>
<td>—</td>
</tr>
</tbody>
</table>

The cells were grown in production medium of Zenk, with a 20% inoculum. dw, Dry weight.

agar and agarose gave consistently lower yield of ajmalicine as compared to the parent. On the other hand cell lines obtained through protoplasts immobilization in agar and agarose produced higher levels of alkaloids as compared to the parent cell line. Cell lines derived through immobilization have been shown to differ in their extent of aggregation and morphology. However, there is no report on the metabolic status of such cells. The present data clearly demonstrates that immobilization of cells and protoplasts bring about marked changes in the alkaloid synthetic pattern of cells emerging through the matrix.

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