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An interchangeable system of hairy root and cell suspension cultures of *Catharanthus roseus* for indole alkaloid production

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Abstract. Hairy roots of *Catharanthus roseus* obtained by co-cultivation of hypocotyl segments with *Agrobacterium rhizogenes*, and cultured in SH (Schenk and Hildebrandt) basal medium, formed two types of calli when subcultured in SH medium with 1 mg/l α-naphthaleneacetic acid and 0.1 mg/l kinetin. One of them, a compact callus, when re-subcultured in SH basal medium gave rise to hairy roots again. A rhizogenic cell suspension culture was established from this type of callus. When cultured in SH medium with growth regulators, the rhizogenic callus produced catharanthine at a level of 41% of the level in the initial hairy roots. Upon transfer to SH basal medium, regenerated hairy roots produced this alkaloid at the original level of 1.5 mg/g dry wt. Using this cell/hairy root interchange system a new management system for hairy root culture in bioreactors has been devised and examined involving production of biomass in the form of a cell suspension in medium supplemented with growth regulators, and catharanthine production by hairy roots regenerated from these cells in medium without growth regulators.

Key words: Catharanthine — *Catharanthus roseus* — Hairy roots — Rhizogenic callus

Abbreviations: NAA, α-naphthaleneacetic acid; SH, Schenk and Hildebrandt; SHNK, SH medium + 1 mg l^{-1} NAA + 0.1 mg l^{-1} kinetin

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Introduction

Hairy roots obtained by *Agrobacterium rhizogenes* infection of stem or root segments have been used as an alternative to cell suspensions for the production of secondary metabolites, due to higher genetic and biochemical stability (Hamill et al. 1987). The long and interlacing structure of hairy roots, however, causes problems regarding transfer to large scale culture facilities and inoculation. Repunte et al. (1993) reported that cell aggregates derived from horseradish hairy roots are capable of regenerating hairy roots which have growth and peroxidase activity comparable to initial hairy roots when repeated through three cycles. This system of cell and root interconversion induced and regulated by exogeneous growth regulator supply can be manipulated to provide convenient seed stock for large scale hairy root cultures. Also, *Hyoscyamus muticus* hairy root cultures produced calli upon treatment with growth regulators, and these hairy root-derived calli did not produce alkaloids at detectable levels. Roots regenerated from such calli, however, showed full restoration of alkaloid synthesis and accumulation (Flores and Filner, 1985). An interchangeable culture system of hairy root and cell suspension of *Catharanthus roseus* is

herein reported where hairy roots regenerated from rhizogenic cells show substantial production of secondary metabolites.

Materials and methods

Hairy roots and culture conditions. Hairy root clone LB1 obtained from seedling segments of *Catharanthus roseus* (L.) G. Don cv. Little Bright Eye infected with *Agrobacterium rhizogenes* 15834 was used (Jung et al. 1992). All cultures were kept at 25℃ in the dark. Suspension cultures were maintained in 300 ml Erlenmeyer flasks containing 50 ml of medium on a gyratory shaker (100 rpm).

Establishment of a cell/root culture system. Callus formation was initiated with 1 cm long tip segments cut from hairy roots and placed onto Schenk and Hildebrandt (SH) (1972) medium solidified with 0.6% Phytagel (Sigma) and supplemented with 1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (SHNK). The competence of the callus to regenerate hairy roots was assessed by transfer of the callus to 1/3 strength SH basal medium. Cell aggregates capable of regenerating hairy roots on this medium were selected, the selection cycle was repeated, and selected cell aggregates were subcultured in SHNK liquid medium to establish cell suspension cultures. These suspension cultures were subcultured every week. To regenerate hairy roots from the rhizogenic callus, calli were inoculated into 1/3 SH basal liquid medium. For a two stage culture from cells to hairy roots, rhizogenic cell suspensions were cultured in SHNK liquid medium for 2 weeks, then the medium was replaced with 1/3 SH basal liquid medium and cultured for another 3 weeks.

Analytical techniques. The solvent extraction procedure and quantitative analysis of indole alkaloids were performed as reported by Jung et al. (1992). An opine test by paper electrophoresis was carried out to confirm the transformed state of initial hairy roots in comparison with non-rhizogenic and rhizogenic calli, and with regenerated and normal roots obtained from seedlings (see Petit et al. 1983).

Results and Discussion

Establishment of a rhizogenic cell line

When root tips were cultured on SHNK medium, two types of calli formed on their surfaces: one was dark-yellow and compact (Fig. 1A), and the other was white and friable (Fig. 1B). When the two types of calli were transferred to 1/3 SH basal medium, roots were regenerated from the dark-yellow, compact callus and grew rapidly like initial hairy roots (Fig. 1C), whereas the white, friable callus stopped growing and was not capable of producing roots (Fig. 1D). Thus, the former was designated as a rhizogenic callus and the latter as a nonrhizogenic callus. Because populations of rhizogenic and nonrhizogenic cells were mixed, selection of rhizogenic cell aggregates was carried out during successive subcultures. After approximately 10 months of subculture and repeated selection, stable rhizogenic and nonrhizogenic cell lines were established.

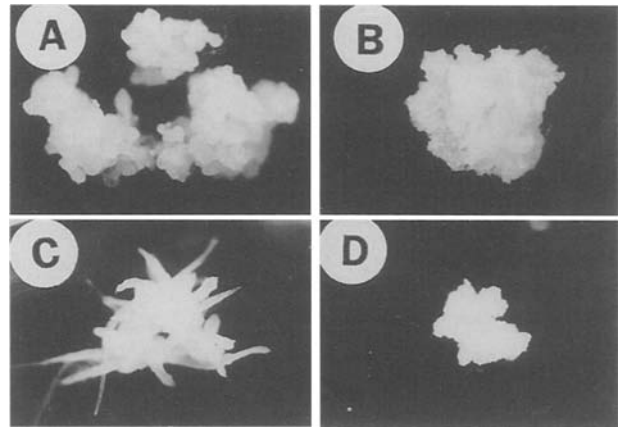

Fig. 1. Morphology changes of cell lines by growth regulators. A, Rhizogenic callus on SHNK medium; B, Nonrhizogenic callus on SHNK medium; C, Hairy roots regenerated from rhizogenic callus on 1/3 SH basal medium; D, Nonrhizogenic callus on 1/3 SH basal medium.

Opine test of cell lines

Each cell line was assayed for opine production by paper electrophoresis. The initial hairy roots transformed by agropine type strain A 15834 produced both agropine and mannopine (Fig. 2, lane A). The rhizogenic calli and corresponding regenerated roots (Fig. 2, lane C, D, respectively) also produced both agropine and mannopine.

However, the non-rhizogenic calli did not produce any opines, suggesting that the transferred T-DNA encoding genes for opine synthesis were deleted or inactive.

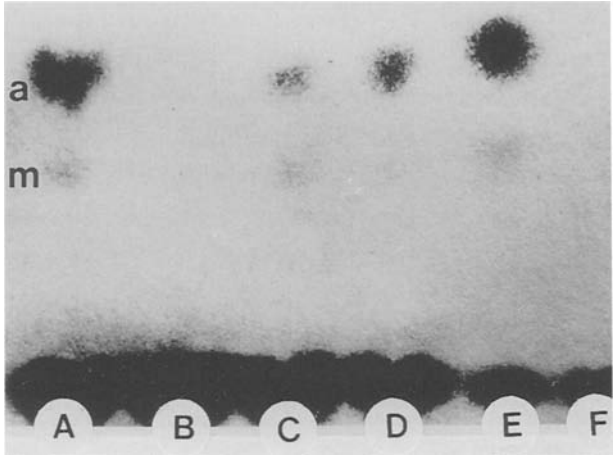

Fig. 2. Electrophoretograms of opines by paper electrophoresis. Lane A, Original hairy roots; B, Nonrhizogenic callus; C, Rhizogenic callus; D, Regenerated roots from rhizogenic callus; E, Standard opines; F, Seedling roots; a, Agropine; m, Mannopine.

Indole alkaloid production by the rhizogenic callus

Rhizogenic cell suspension cultures were established from rhizogenic calli after successive subcultures in SHNK liquid medium. The catharanthine content of rhizogenic calli was determined at different growth stages (Fig. 3). Hairy root LB1 produced catharanthine at a level of 1.7 (mg/g dry wt) and its productivity was retained for over two years. When rhizogenic calli were cultured on SHNK liquid medium, the catharanthine content decreased to 41% of that of initial hairy roots. However, after 3 weeks of inoculation of rhizogenic calli into 1/3 SH basal medium, roots were regenerated from the calli and the catharanthine content was restored to nearly the same level as the initial hairy roots.

To examine the possibility of high density culture by a two stage culture system from cells to hairy roots, rhizogenic cell suspensions were cultured in SHNK liquid medium for 2 weeks (Fig. 4A). When the medium was replaced with

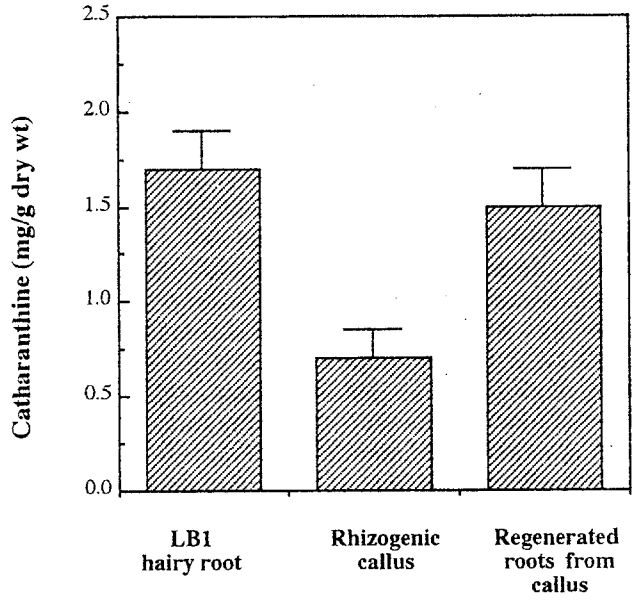

Fig. 3. Effects of morphological changes on catharanthine production in hairy root cultures. The vertical bars indicate s.e.

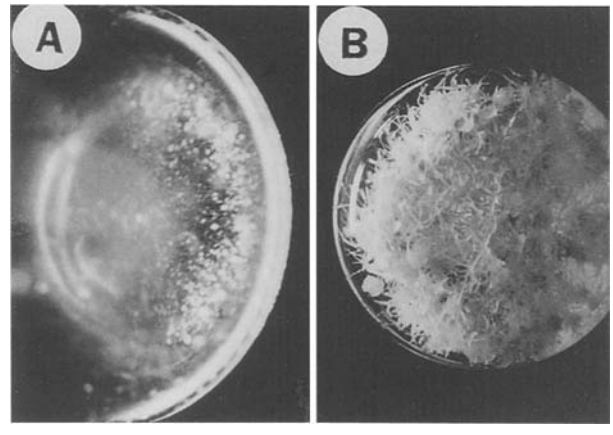

Fig. 4. Regeneration of hairy roots by two stage culture. A, Initial state of rhizogenic callus in SHNK liquid medium; B, After 3 weeks of culture in 1/3 SH basal liquid medium.

1/3 SH basal liquid medium and cultured for 3 weeks, hairy roots were generated and they proliferated until they were densely packed in the culture flask (15 g dry wt l^{-1}) (Fig. 4B). The catharanthine content reached 1.4 mg/g dry wt and the production of other indole alkaloids was also restored (data not shown).

An interchangeable hairy root/suspension culture system

Secondary metabolite production seems to be closely linked to morphological differentiation and is generally suppressed when cells proliferate actively (Berlin et al. 1980). 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 indefinitely 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 *H. 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, in this study, 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. Furthermore, the reverted hairy roots also regained productivity of the secondary metabolites of interest.

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.

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