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# IMPROVEMENT OF THE CATHARANTHINE PRODUCTIVITY IN HAIRY ROOT CULTURES OF *CATHARANTHUS ROSEUS* BY USING MONOSACCHARIDES AS A CARBON SOURCE

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**SUMMARY** ; Sucrose, glucose, and fructose as carbon sources in culture medium were assessed in hairy root cultures of *Catharanthus roseus*. The cultures preferentially consumed sucrose, resulting in about 40% (dry wt) higher growth rate. However, fructose enhanced the cathranthine yield about two-fold. The elevated yield was not seemingly ascribed to the higher osmolarity per unit weight of fructose than sucrose. A two stage culture using sucrose (1st) and fructose (2nd) improved volumetric yields of catharanthine about two-fold, i.e. 41 mg/l.

## INTRODUCTION

*Catharanthus roseus* plants produce commercially important anticancer agents such as vinblastine and vincristine. Vinblastine and vincristine are formed by coupling two different monomeric indole alkaloids, vindoline and catharanthine. In the plant, the former is accumulated at a relatively high level, whereas the latter is at a much lower level. Therefore, it is necessary to develop efficient *in vitro* catharanthine production system to couple with vindoline extracted from the cultivated plants for vinblastine and vincristine production.

Hairy root cultures obtained by infection by *Agrobacterium rhizogenes* have advantages over cell suspension cultures for production of valuable secondary metabolites due to their genetic and biochemical stability. In addition, it is possible to select hairy root clones which grow faster and produce more secondary metabolites of interest (Hamill *et al.*, 1987).

Sucrose or glucose have been found to be effective in supporting high growth rates and biomass yields in plant cell cultures (Fowler and Stepan-Sarkissian, 1985). In the case of hairy root cultures, usually, sucrose is used as a carbon source (Toivonen *et al.*, 1989 ; Hilton and Rhodes, 1990). However, recently, Uozumi *et al.* (1991) reported that fructose is a better carbon source for high density culture of carrot hairy roots. There has been no report on the assessment of carbon sources on improvement of indole alkaloid production in *C. roseus* hairy root cultures.

## MATERIALS AND METHODS

Induction of hairy roots.

To induce hairy roots, 1 cm long hypocotyl segments of seedling of vinca (*Catharanthus roseus* (L.) G. Don; cv. Little Bright Eye) were infected with *A. rhizogenes* strain 15834 and cultured on Shenk and Hildebrandt (1972) basal medium (SHBM). Hairy roots formed on the segments were excised and cultured on 1/3 SHBM (one third dilution of SH basal salts) supplemented with 0.5 g carbenicillin/l. Rapidly growing hairy roots without bacterial contamination were subcultured 2-3 times on the medium and transferred into the same component of liquid medium. Among the hairy roots, a clone showing a high growth rate and yield of indole alkaloids was selected for further experiments. The opine test was carried out to confirm its transformation (Petit *et al.*, 1983).

Culture of hairy roots.

The hairy roots were subcultured in S medium (1/3 SH liquid medium containing 30 g sucrose/l) every three weeks. To assess carbon sources, 0.3 g (fresh weight) of hairy roots were inoculated into 30 ml of F medium (30 g fructose/l) and G medium (30 g glucose/l) in a 100 ml Erlenmeyer flask. Either sucrose, glucose or fructose was added to pre-autoclaved 1/3 SH liquid medium (pH 5.7) through a membrane filter (pore size, 0.2 μm). All cultures were maintained in the dark at 25°C on a gyratory shaker (100 rpm). For the two stage culture, after 15 days of culture in S medium, the old medium was replaced with fresh F medium. The hairy roots were harvested and washed once with double-distilled water before measuring the weight. Dry weight was measured after desiccation at 60°C for 24 h.

Analysis of indole alkaloids.

The solvent extraction procedures and quantitative analysis of indole alkaloids were performed by the method of Jung *et al.* (1992). For each sample, 50 mg (dry wt) of hairy roots was extracted three times in an ultrasonic bath at 50°C with 10 ml methanol for 30 min. Following the solvent extraction, the alkaloid extract was loaded onto a reversed phase column, μ-Bondapak $C_{18}$ column (3.9 x 300 mm) connected with Spectra-Physics HPLC system. The solvent mixture of methanol, acetonitrile and diammonium hydrogen phosphate (pH 7.3, 3/4/3, by vol) was eluted at a flow rate of 1 ml/min, and indole alkaloids were detected at 298 nm. The quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic indole alkaloids. The qualitative analysis of indole alkaloids produced from the hairy roots was carried out by mass spectrometer (Hewlett-Packard 5989A Mass Spectrometer ; 150°C ion source temperature and 70 eV).

Analysis of carbohydrates.

The concentrations of sucrose, glucose, and fructose in culture medium were measured using an Aminex HPX-87C column (300 X 7.8mm, Bio-Rad) connected with Beckmann HPLC system with a refractive index detector. The column temperature was kept at 90 °C during the analysis. The HPLC grade water was eluted at a flow rate of 0.5 ml/min.

## RESULTS AND DISCUSSION

Establishment of hairy root cultures.

About 10% of the hypocotyl segments protruded one or two hairy roots after 2 - 3 weeks of culture. Among the hairy roots, a clone coded LB1 showed a relatively high growth rate and yield of indole alkaloids (Fig. 1), which was selected for further experiments. Production of agropine and mannopine was detected in the LB1 hairy roots by paper electrophoresis (data not shown), indicating that it was transformed by the bacterial strain.

Indole alkaloid production.

A typical HPLC chromatogram of indole alkaloids produced by LB1 was obtained (Fig. 2). Two compounds detected at 10.44 and 8.44 min (retention time) were putatively identified as catharanthine and ajmalicine, respectively, when compared with those of the authentic compounds. The mass spectra of catharanthine and ajmalicine produced from LB1 were the same as those of the authentic samples (data not shown). The catharanthine yield (1.9 mg/g dry wt) of LB1 was as high as that of other reported lines (Toivonen *et al.*, 1989; Fujita *et al.*, 1990). The ajmalicine yield was somewhat lower (0.8 mg/g dry wt).

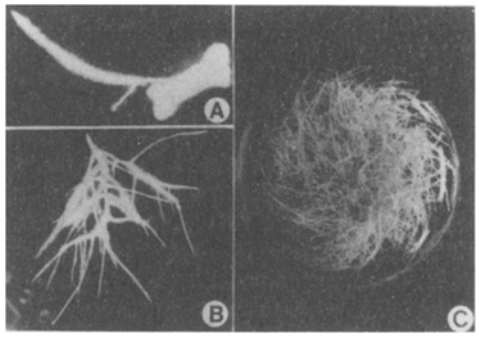


**Figure 1. Establishment of *C. roseus* hairy root culture.**
**A : Formation of hairy root on hypocotyl segment**
**B : Proliferation of hairy root from one root tip.**
**C : Hairy root cultured in an Erlenmeyer flask.**

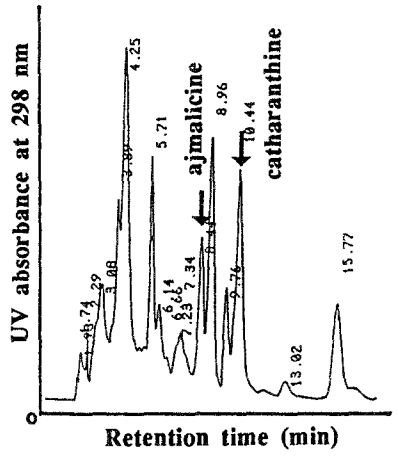


**Figure 2. HPLC chromatogram of alkaloids produced in *C. roseus* hairy root cultures.**

Growth kinetics using sucrose as a carbon source.

Figure 3 shows the growth kinetics and carbohydrates consumption of the hairy root culture using sucrose. The sucrose concentration was reduced in inverse proportion to the increment of biomass, whereas the monosaccharides hydrolyzed from sucrose increased during the lag phase and subsequently maintained a plateau. The utilization of carbohydrates was considerably different from that in cell suspension cultures. In cell suspension cultures, sucrose is rapidly and completely hydrolyzed to glucose and fructose during the early log phase, and then hydrolyzed glucose is preferentially utilized over fructose by the cells (Nikolova *et al.*,1991).

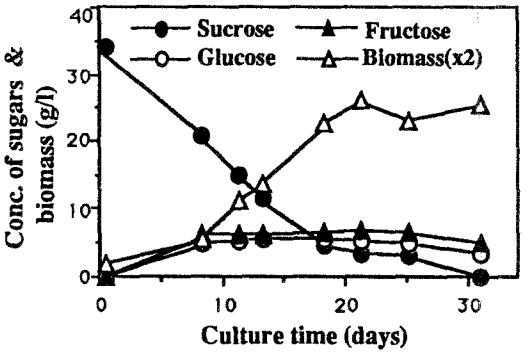


**Figure 3. Profile of the sugar consumption and growth of *C. roseus* hairy root in 1/3 SH medium containing 30 g sucrose/l.**

Kinetics of growth and catharanthine production using monosaccharides.

The effects of monosaccharides on the growth and catharanthine production in the LB1 culture are shown in Figure 4. When glucose or fructose was used as a carbon source, the lag phase was slightly extended and the specific growth rate ($Day^{-1}$) was lower than with sucrose. The specific growth rate ( $\mu$ max, s) in S medium was 0.21 $D^{-1}$, and the specific growth rates in G ( $\mu$ max,g) and F ( $\mu$ max,f) media were 0.14 and 0.13 $D^{-1}$, respectively. There was little difference in growth kinetics between G and F media (Fig. 4-A). The maximum biomass (Xmax) in monosaccharide medium reached about 70% of that in S medium. Although the growth rates were different, biomass yields (biomass/consumed sugar, g/g) were almost the same in the three media. The yield coefficients of biomass for S (Yx/s), G (Yx/g), and F (Yx/f) media, were 0.53, 0.50, and 0.49, respectively. But, in respect of product yield (Yp/x, catharanthine /biomass, mg/g) , there was an enormous difference between S and G or F medium. As shown in Figure 4-B, use of F medium increased the yield of catharanthine of the hairy root up to about two-fold (3.5 mg/g dry wt) that in S medium (1.9 mg/g dry wt). This phenomenon was probably ascribed to the following. First, the osmolarity of the same weight of monosaccharides is about twice than that in S medium, which may stimulate secondary metabolite production as shown in cell suspension cultures of *C. roseus* ( Merillon *et al.*, 1984). Second, replacement of sucrose by glucose or fructose may change the cellular carbohydrate metabolism, and subsequently stimulate the production of the compound.

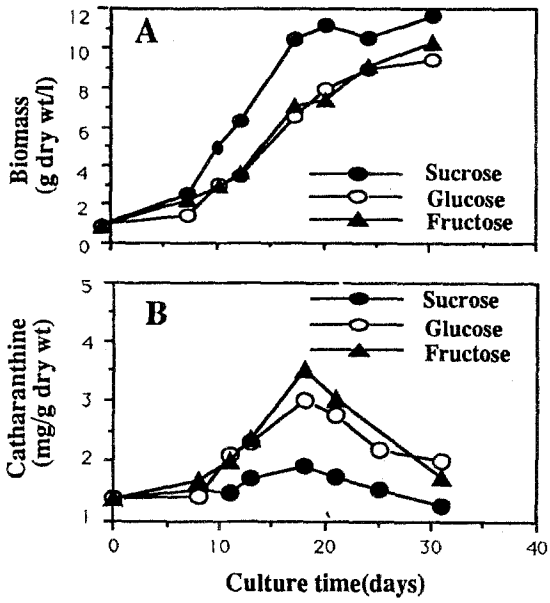


**Figure 4. Growth and catharanthine accumulation of *C. roseus* hairy root cultures using different sugars. Hairy roots were cultured in medium containing either sucrose, glucose or fructose at 30 g/l.**
**A : profile of hairy root growth**
**B : profile of catharanthine yield in hairy root.**

To investigate the effect of osmolarity on alkaloids production, hairy roots were cultured in either F, S, or S45 (45 g sucrose /l) medium. Their growth kinetics are shown in Figure 5-A, in which the growth rate in S45 medium was higher than that in S and F media. As the osmolarity of the medium is mainly proportional to the concentration (molarity) of the sugars, we analyzed the changes in the sugar concentrations during the

culture periods. The initial concentrations of sugars in F, S, and S45 medium were 166, 88, and 132 mM, respectively. As shown in Figure 5-B, the osmolarity of S45 was kept higher than F medium after the early exponential phase because of continuous hydrolysis of sucrose into glucose and fructose. On the contrary, the yield of catharanthine of hairy root in F medium was higher than those of S45 and S media (Fig.5-C). This result suggests that the high osmolarity of monosaccharides over sucrose does not directly cause the increase of catharanthine production.

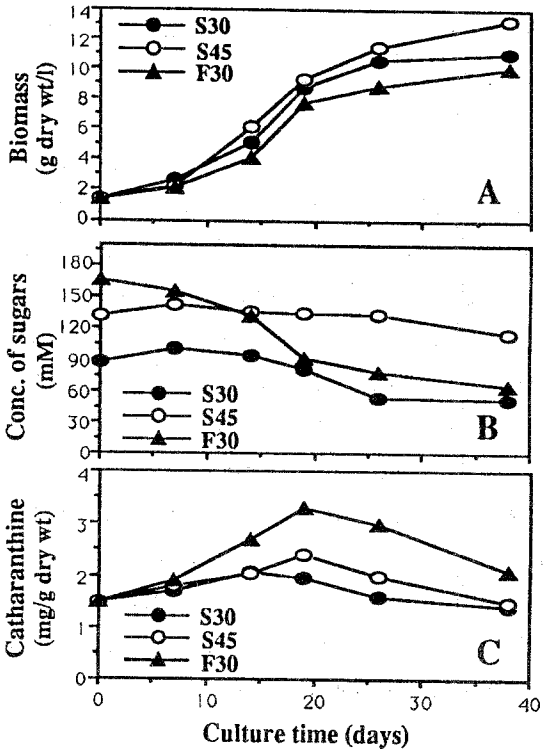


**Figure 5. Changes in Biomass (A), molarity of sugars (B), and catharanthine yield (C) in three different culture media containing either 30 g sucrose/l (S30), 45 g sucrose/l (S45) or 30 g fructose/l (F30) during *C. roseus* hairy root culture.**

Two stage culture from sucrose to fructose medium.

Even though the catharanthine yield in F medium was higher than that in S medium, maximum biomass in F medium was lower. Thus, to increase the volumetric yield, we attempted two stage culture using sucrose (1st) and fructose (2nd) as a carbon source. Little difference in the hairy root growth was noticeable between one stage culture in S medium and two stage culture (Fig.6-A). However, two stage culture markedly increased volumetric yields of catharanthine about two-fold (41 mg/l) than that (20 mg/l) of one stage culture in S medium

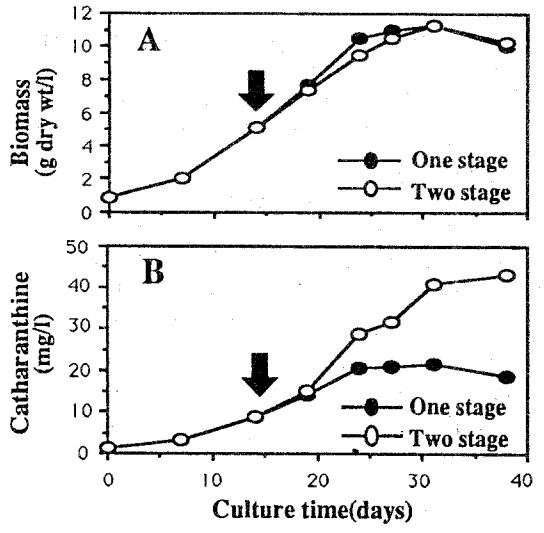


**Figure 6. Comparison of the growth and catharanthine production between one stage and two stage culture of *C. roseus* hairy root.**
**Arrows indicate the time of medium change from medium containing sucrose to fructose at 30 g/l.**
**A : profile of hairy root growth**
**B : profile of catharanthine production.**

due to the increased yield of catharanthine per g dry wt of hairy roots (Fig. 6-B).

Mechanism of sucrose uptake into the cells can broadly be divided into two categories, hydrolytic and non-hydrolytic process. In the former, glucose and fructose hydrolyzed from sucrose by external invertase are taken up by the cell. But, in case of the latter, sucrose is directly taken up by the cell in the form of the intact disaccharide. In this study, consumption rate of sucrose in the culture was faster than monosaccharides, indicating that the mechanism of sucrose uptake in hairy root culture is mediated by both the hydrolytic and non-hydrolytic process. When glucose or fructose was substituted for sucrose, the specific growth rate lowered, but the yield of catharanthine increased up to about two-fold. As mentioned above, it did not seemingly result from the high osmolarity of monosaccharides over sucrose.

We suggest that the substitution of fructose for sucrose alters carbohydrate metabolism in hairy roots, which subsquently stimulates their secondary metabolism. In this study, somehow, we demonstrated a marked improvement of catharanthine yield in hairy root cultures by monosaccharides over sucrose. The two stage culture system amplifying the monosaccharides effects is currently under scale-up process development.

## ACKNOWLEDGEMENTS

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