

Enhancement of Catharanthine Production in *Catharanthus roseus* Cell Cultures by Adding Tryptophan as Precursors

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Abstract

Feeding precursor has a role on the secondary metabolite enhancement in *in vitro* culture. The effect of tryptophan on catharanthine production was studied in aggregate cells of *Catharanthus roseus* (L.) G Don. Aggregate cells of *C. roseus* were cultured in Murashige and Skoog (MS) medium supplemented with 2 ppm NAA and 0 ppm kinetin. Aggregate cells culture were divided into control and treatment groups and were fed by 100, 125, 150, 175, 200, 225 mg/L tryptophan, respectively. The growth of cells and the catharanthine production in the aggregate cells as well as in medium were observed on the 1st and 10th days. Qualitative and quantitative analysis were conducted using High Performance Liquid Chromatography (HPLC) in VP-ODS C-18 column, eluted using methanol : acetonitrile : diammonium hydrogen phosphate (3 : 4 : 3), rate 1 ml/minute. The results showed that catharanthine was detected in the aggregate cells and culture medium. The highest concentration of catharanthine in the aggregate cell and the medium were obtained on the addition of 175 mg/L tryptophan, i.e. 1650.48 ug/g and 10329.75 ug/L respectively. Tryptophan feeding enhanced catharanthine content in aggregate cell by 171.02%, as well as its secretion into medium by 329.34%.

Keywords : Catharanthine, Tryptophan, *Catharanthus roseus*, aggregate cells

1. Introduction

Plants provide not only materials for foods, wood, etc. but also a wide range of chemical compounds, which perform a variety of functions such as flavours, fragrance, colours and insecticides. These compounds belong to a group collectively known as secondary products (metabolites). It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained either from or modeled on plant substances (Tripathi & Tripathi, 2003).

The Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don), is characterized by the large variety of monoterpenoid indole alkaloids that it produces. Among these many structures, vinblastine and vincristine are of particular importance because of their wide use in cancer chemotherapy. These alkaloids are produced *in vivo* by the condensation of vindoline and catharanthine. The pharmaceutical value of these dimeric alkaloids, their low abundance, and their cost of production have prompted extensive efforts to generate cost efficient high-yielding methods.

Cell suspension cultures, in particular, have received considerable attention due to their rapid growth, relatively simple cultivation

requirements, and amenability for scale-up (Facchini and DiCosmo, 1991). Reports from several experiments showed that few successful industrial applications are introduced due to several biological and technological limitations, particularly the low yield of these compounds in cell cultures (Zhao *et al.*, 2001, El-Sayed *et al.* 2004). The optimization of medium composition, including the supply of biosynthetic precursors, is among the various strategies that have been followed to increase alkaloid production *in vitro* (Moreno *et al.*, 1993).

The precursors for the synthesis of terpenoid alkaloids are obtained from tryptamine and secologanin. Tryptamine is synthesized from Tryptophan. The condensation of tryptamine and secologanin form strictosidine, as a key step to produce alkaloids, such as catharanthine and vindoline. Therefore, addition of tryptophan can be crucial to increase catharanthine production. The objective of this experiment was to evaluate the effect of various tryptophan concentration on catharanthine production in aggregate cells of *c.roseus* and its secretion into the surrounding medium.

II. Materials And Methods

Medium preparation

Murashige and Skoog (MS) medium was used for both, in callus and cell aggregate culture. For the experiment, Tryptophan was added into the cultures medium to give a concentration of 100, 125, 150, 175, 200, and 225 mg/L respectively per flask. The controls received the same amount of distilled water.

Sample materials

Catharanthus roseus cell suspension culture was established from leaf explants on MS medium. The initiation medium contained 1.5 ppm 2,4-D and 0.15 ppm kinetin, whilst production medium contained 2 ppm NAA and 0.2 ppm kinetin. The cell suspension was initiated by transferring the compact callus into the liquid medium. It was transferred into the same conditions (Control) or it was transferred into the medium with the addition of tryptophan as the precursor (Treatment : 100, 125, 150, 175, 200, 225 ppm), and sub-cultured every two weeks. Cultures were shaken at rotary shaker: 120 rpm and incubated at 25 °C, with 24 h light. The experiment was performed in triplicate for 10 days. Sampling was done at the beginning (d-1) and the end of the experiment (d-10).

Sample extraction and analysis of catharanthine.

Catharanthine was extracted from cell aggregates and medium according to the modification of method described in Lee *et al.*(1981 in Asada & Shuler, 1989). HPLC analysis was performed using a shimpak VP-ODS C18 (0.15 x 0.6). The solvent system used was methanol : acetonitrile : diamonium hydrogen phosphate (3 : 4 : 3), isochratically. The samples were injected at 1.0 mL min⁻¹. Peak identification was based on a comparison of retention time with authentic catharanthine standard

III. Results And Discussion

Aggregate Culture establishment

Callus was first observed one week after the leaf explant was cultured in MS medium supplemented with 1.5 ppm 2,4 D and 0.15 ppm kinetin (see fig.1). In order to produce high yielding catharanthine line, callus was transferred into MS medium supplemented with 2 ppm NAA and 0.2 ppm kinetin so that it produced compact callus. Dark and compact

callus usually contain higher alkaloid, such as catharanthine, vindoline, ajmalicine, and serpentine (Morris, 1986 in Fowler and Warren, 1992). Compact callus was transferred into liquid medium to form cell suspension culture. According to Verpoorte *et al.* (2000), the use of suspension culture of *C.roseus* had an advantageous, i.e. high tryptophan decarboxylase activity in regulating biosynthesis of tryptophan, as precursor in monoterpene indol alkaloid biosynthesis. The type of cell suspension culture was aggregate cells (see fig.2).

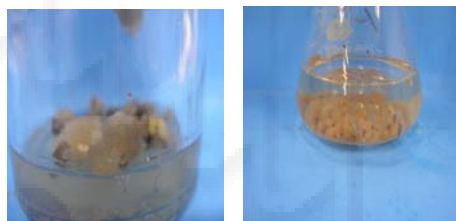


Fig.1 Callus

Fig.2 Cell Aggregate

Molecular aggregation which create conditions that more closely resemble those of organized plant tissue are more beneficial, because cellular confluence may result in the establishment of biochemical gradients, a greater capacity for intercellular communication, and a cyto-and/or biochemical-differentiation (Lindsey and Yeoman 1983). This result were also observed by Zhao *et al* (2001) by using compact callus clusters (CCC) of *C. roseus*. Plant growth regulators and sucrose concentrations significantly influenced the size, the degree of compaction, and alkaloid accumulation of the CCC cultures, therefore when 2,4-D was replaced with NAA or IAA, both in light and in dark, callus synthesized some vindoline and catharanthine as well as high levels of ajmalicine and serpentine.

The growth of the cell cultures

In all the experiments, addition of various concentrations of precursors (tryptophan) affected the growth of the cell cultures, as determined by measuring the amount of biomass obtained at the end of each experimental period, which differs in each experiment on dry-weight basis by 6.62 – 60.31 % (see Fig 3). Among the cultures, addition of 175 ppm tryptophan gave the highest dry weight(DW) at day 10 compared to control. Although all treatment caused increment to the growth of cells, the effect of feeding precursors on growth varies depending on the cell line and the type of precursor (El Sayed *et al*, 2002). Moreno *et al.* (1993) found

no effect of loganin feeding (0.5 mM) on the same cell line, and combined feeding of 1 mmol of loganin and tryptamine decreased the growth compared with non-fed cells. Moreover, no effect of loganin/tryptamine feeding was found on transgenic cell line T22 at lower concentrations.

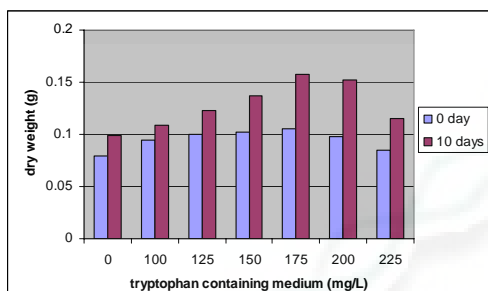


Fig.3 Cell aggregate growth

Tryptophan has an indirect role on the growth via its influence on auxin synthesis. Available evidence suggested that several alternative routes of IAA synthesis, in plants, were all starting from amino acids, including tryptophan (Dahab & El-azis, 2006). It was suggested that increased endogenous IAA would induce cell division, which then increased the capacity of tissue to grow. Verpoorte *et al.*(2000) found that addition of tryptophan could over-express tryptophan decarboxylase (TDC) enzymes activity, followed by the increment of fresh weight and dry weight of suspension cell culture.

Statistically, (95 %), addition of 175 mg/L tryptophan increased 60.42 % of cell DW compared to control, but addition of higher concentration of tryptophan (200 & 225 mg/L) only resulted in 54.26 & 16.42 % increment, respectively. This might be the result of precursor toxicity (Anita & Kumari, 2006). Canel *et al.* (1989) also observed that over-expression of TDC in the callus and cell of *C.roseus* gave a sign of stress and so reduced growth of callus and cell.

Alkaloid accumulation

A lower concentration of catharanthine was observed in the non-fed samples, which might be as a result of deficiency in endogenous precursor production, whilst feedings on tryptophan caused higher catharanthine accumulation in cell (Figure 4) both in day 1 and 10. However, catharanthine accumulation on d 10 showed much higher catharanthine than d 1.

The cells quite efficiently (about 8 -53 to 131-171% respectively) converted tryptophan into catharanthine after 10 days feedings. However, the highest accumulation of catharanthine in the cells (1650.48 ug/g DW) was found in the addition of 175 mg/L tryptophan. High catharanthine accumulation might occur through high TDC activity which catalyzed the conversion of tryptophan into tryptamines, followed by induction of strictocidine syntase (STR) activity to condense tryptamine and secologanin into strictocidine, as a precursor of all indole alkaloids, including catharanthine (Canel *et al.*,1998).

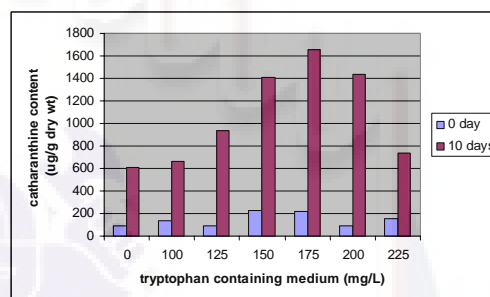


Fig. 4 Catharanthine content in the cell

Addition of higher tryptophan, i.e .200 and 225 mg/L, resulted in smaller amount of catharanthine accumulation in the cell. It thus seems that the level of catharanthine accumulation is controlled by factors other than availability of tryptophan, or suggests a rate limiting step in the conversion of tryptophan into catharanthine, resulting in channeling of tryptophan into other unknown products. This results were also observed by others (Canel *et al.*, 1998; Zhao *et al.*,2001; Anita & Kumari, 2006), in which the ability to respond to precursors depended on the characteristic of genetic and physiology of the cells. Furthermore, precursor level of toxicity could become a limiting factor in the conversion of tryptophan into catharanthine.

It was observed that the most of the increased amounts of alkaloids could be recovered from the medium (figure 5.). The additions of tryptophan significantly stimulated catharanthine production, and up to 329.34% was released into the medium. Nijkamp *et al* (1990) also observed that some metabolites produced in the cells were released (secreted) into the surrounding medium with active or passive mechanism, whilst Neumann (1983) suggested that alkaloids released was triggered

by pH change. Therefore, feedings of tryptophan enhanced indole alkaloid production in the aggregate cultures and promoted release of catharanthine into the medium. Result which was in agreement with results of Zhao *et al.*(2001). However, cultures with different genetic and physiological characteristics respond differently to the supply of precursor for indole alkaloid biosynthesis.

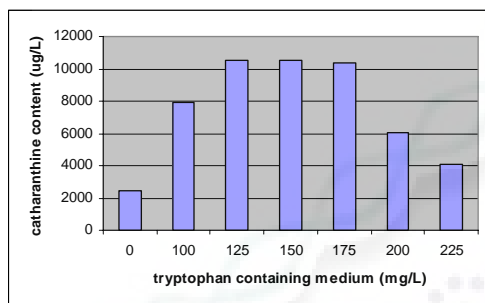


Fig.5 Catharanthine content in culture medium

IV. Footnotes

This work was partially supported by a grant from the Joint Research PEKERTI with Pandiangan, Sam Ratulangi University, Indonesia

V. Reference:

1. S Anita, B.D.R. Kumari. Stimulation of reserpine biosynthesis in the callus of *Rauwolfia tetraphylla* L. by precursor feeding. *African Journal of Biotechnology* Vol. 5(8), 659-661(2006).
2. C Canel, M. I Lopes-Cardoso, S Whitmer, L.van der Vits, G Pasquali, R van der Heijden, J. H. C. Hoge, R Verpoorte.. Effects of over-expression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. *Planta* 205: 414-419 (1998).
3. D Neumann, G Krauss, M Hieke, G Groger. Indole Alkaloid Formation and Storage in Cell Suspension Cultures of *Catharanthus roseus*. Dalam : *Planta Medica*. 48 : 20-23 (1983).
4. H.J.J.Nijkamp, L.H.W. van der Plas, J.van Aartijk, Progress in Plant Cellular and Molecular Biology, Current Plant Science and Biotechnology in Agriculture. Kluwer academic Publisher, Netherlands.(1990)
5. J. Zhao, Q. Hu, Y.-Q. Guo, W.-H. Zhu, Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters

6. J. Zhao, W-H Zhu, Q Hu, Effects of light and plant growth regulators on the biosynthesis of vindoline and other indole alkaloids in *Catharanthus roseus* callus cultures, *Plant Growth Regulation* 33: 43-49 (2001).
7. K Lindsey, M M. Yeoman. Novel Experiment System for Studying The Production of Secondary Metabolites by Plant Tissue Culture. Cambridge Univ Press.London (1983).
8. M.Asada, ML Shuler. Stimulation of Ajmalicine Production and Excretion from *Catharanthus roseus* : Effect of Adsorption *in situ* Elicitor and Alginate immobilization. In: *Applied of Microbiology Biotechnology*. 30 : 474 – 481 (1989).
9. M El-Sayed, YH. Choi1, M. Frederick, S.Roytrakul, R. Verpoorte, Alkaloid accumulation in *Catharanthus roseus* cell suspension cultures fed with stemmadenine , *Biotechnology Letters* 26: 793-798 (2004).
10. M W Fowler, G S Warren. *Plant Biotechnology : Comprehensive Biotechnology, second Supplement*. Pergamon Press plc. England, (1992)
11. P J. Facchini, F DiCosmo, Secondary metabolite biosynthesis in cultured cells of *Catharanthus roseus* (L.) G. Don immobilized by adhesion to glass fibres, *Appl Microbiol Biotechnol* 35:382-392 (1991).
12. P. R. H., Moreno, Van der Heijden, R. Verpoorte. *Influence of Stress Factors on The Secondary Metabolites in Suspension Cultured Cells*. Brazil. (1993).
13. R. Verpoorte, , R. van der Heijden, J. Memelink.. Engineering the plant cell factory for secondary metabolite production. *Transgenic Research* 9: 323-343 (2000).
14. T.A.M Dahab, N. G Abdul-azis. Physiological Effect of Diphenylamin ang Tryptophan on the Growth and Chemical Constituents of *Philodendron erubescens* Plants. *World Journal of Agricultures Sciences* 2 (1): 75-81 (2006).
15. L.Tripathi, J N.Tripathi, Role of biotechnology in medicinal plants, *Tropical Journal of Pharmaceutical Research*, 2(2): 243-253 (2003).