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Influence of an Indirect Precursor on Podophyllotoxin Accumulation in Cell Suspension Cultures of *Podophyllum hexandrum*

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Abstract

Podophyllum hexandrum is an endangered, commercial source of a naturally occurring lignan podophyllotoxin, which is a precursor of potent anticancer drugs. Cell suspension cultures were established using four-year-old cell line RC4 of *P. hexandrum* in liquid P1 medium [half strength B5 basal medium supplemented with 1% (w/v) sucrose, 2,4-D (1 mg/l), GA_3 (1 mg/l) and BA (0.1 mg/l)]. The effect of different concentrations (100, 250 and 500 mg/l) of tryptophan, an indirect precursor of lignan biosynthesis, was studied on cell suspension cultures. Cultures accumulated 2.7 times more podophyllotoxin than untreated control cultures when treated with 250 mg/l tryptophan. Biomass yield was, however not improved following the addition of tryptophan.

Introduction

Podophyllotoxin (an aryl tetralin lignan) is a pharmaceutically active natural compound. It is widely used as a precursor for the semisynthesis of well-known antitumour drugs like etoposide (VP-16-213) and teniposide (VM-26) which are used in the treatment of lung cancer, testicular cancer, a variety of leukemias and other solid tumours (Holthius 1988, Stahelin and Wartburg 1991, Imbert 1998). But the availability of podophyllotoxin has become limited due to the dwindling nature of its commercial source *Podophyllum hexandrum* (Indian Podophyllum, Berberidaceae) which has already been categorized as a threatened species and low yielding, uneconomical chemical synthesis of the compound. The limited supply of podophyllotoxin from *P. hexandrum* has prompted intense efforts to develop alternate sources and means of production. Biotechnological approaches for the production of podophyllotoxin were first reported by Kadkade (1981) in

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P. peltatum (American Podophyllum) and by Uden et al. (1989) in *P. hexandrum* callus cultures. Since then several studies have been carried out for improvement of podopyllotoxin accumulation in cell cultures of *P. hexandrum* (Uden et al. 1990, Woerdenbag et al. 1990, Chattopadhyay et al. 2002, 2003, Lin et al. 2003). The author has also successfully initiated and maintained cell cultures of *P. hexandrum* for several years in their laboratory (Majumder and Jha 2009). The objective of the present study was to improve podophyllotoxin accumulation in cell suspension cultures of *P. hexandrum* through addition of tryptophan, an indirect precursor of lignan biosynthesis.

Materials and Methods

Cell suspension cultures of four-year-old friable cell line RC4 (Majumder and Jha 2009) were initiated and established in 20 ml liquid P1 medium [half strength B5 basal medium (Gamborg et al. 1968) supplemented with 1% (w/v) sucrose, 2,4-D (1 mg/l), GA_3 (1 mg/l) and BA (0.1 mg/l)] in 150 ml flasks. Cultures were incubated in dark at 100 rpm on a gyratory shaker at 24 ± 1ºC with relative humidity of 55 -60% and subcultured after a period of 14 days by filtering through sterile mesh (60 μm) to obtain finely suspended cells. Larger cell aggregates were removed. A volume of 5 ml of inoculum (~10^4 cells/ml) was transferred to 20 ml fresh medium (1 : 4) in a 150 ml flask. Cultures were maintained by regular subculturing at two weeks interval. Samples were collected from finely dispersed cell suspension cultures and regularly examined under inverted microscope (Model: LABOVERT, Leitz). Cell density was estimated and monitored by using a hemocytometer.

Different concentrations (100, 250 and 500 mg/l) of filter sterilised tryptophan were added to 14-day-old cell suspension cultures established with four-year-old cell line RC4 as mentioned above and grown at 100 rpm on a gyratory shaker at 24 ± 1ºC in the dark with relative humidity of 55 - 60%. After seven days cultures from each flask were dried (48 hrs, 45°C) and weighed after harvesting cells on a Buchner funnel applied with a filter paper for analysis of cell growth and podophyllotoxin content. Growth index (GI) was expressed as a ratio of final and initial dry weights (DW) of the cell masses. Ten replicates were used for each treatment and all experiments were repeated thrice.

Podophyllotoxin was extracted from cultured cells following the method of Heyenga et al. (1990) with modification and analysed by HPLC using standard samples of podophyllotoxin (Sigma). Samples (0.1 - 0.2 gDW) were heated with dehydrated ethanol on water bath for 3 hrs after which the ethanol phase was evaporated to dryness. Extracts of cell free medium were prepared by mixing with chloroform (1 : 2) and stirred continuously for 3 hrs using a magnetic stirrer.

The chloroform fractions were separated using a separatory funnel and dried. The dried residues were redissolved in 1 ml analytical grade methanol, filtered (Sartotius filters, 0.22 μm) prior to analysis and analysed by HPLC as reported earlier (Majumder and Jha 2009). For quantitative analysis peak areas were used to calculate the amount of podophyllotoxin present in the cultured cells and these were compared to the standard. The standard sample was used to construct a calibrated graph by plotting peak areas versus amount of podophyllotoxin injected over a range of 5 -40 μg. The relationship was linear over ten measurements. Identification of podophyllotoxin was done by retention time and spiking with standard (Majumder and Jha 2009). Podophyllotoxin was identified by preparative HPLC and identified using ^{1}H-NMR by comparing with literature values (Jackson and Dewick 1984a).

Data were analysed by ANOVA to detect significant differences between means (Sokal and Rohlf 1987). Means differing significantly were compared using the Duncan (1955) DMRT at the 5% probability level. Variability around the mean was represented as ± the standard error.

Results and Discussion

Growth kinetics of cell suspension culture of RC4 showed a typical growth pattern characteristic of cell suspensions (Fig. 1). Cell counting with hemocytometer revealed that there was rapid growth during the first phase of the growth cycle (day 0 to day 14) followed by an inhibition of growth in the second phase (day 14 to day 21). Maximum cell density ($13.4 \pm 0.5 \times 10^4$ cells/ml) was noted on day 14 after which there was a sharp decline in cell density ($10 \pm 1 \times 10^4$ cells/ml on day 21). Thus, tryptophan was added on the 14th day of the growth cycle and cells harvested seven days later.

The effect of tryptophan on biomass yield and podophyllotoxin accumulation in cell suspension cultures of RC4 was studied. In untreated cell suspensions, podophyllotoxin content was 2.8 ± 0.5 mg/gDW. Suspension cultures accumulated 2.7 times more podophyllotoxin than untreated control cultures when treated with 250 mg/l tryptophan (Fig. 2). But higher (500 mg/l) or lower (100 mg/l) concentrations of tryptophan used could not improve podophyllotoxin accumulation in cell suspension cultures. Podophyllotoxin was not detected in any of the culture media analysed indicating that it was only present intracellularly and not exuded in the culture medium. However, cell growth decreased after the addition of tryptophan.

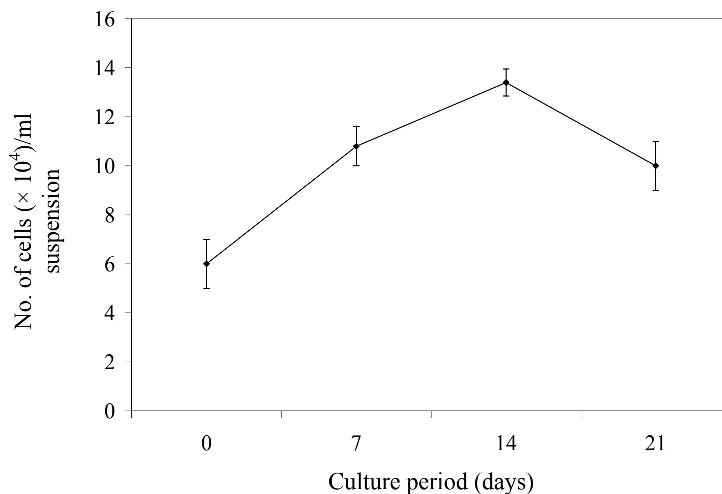

Fig. 1. Growth kinetics of cell line RC4 cultured in 20 ml liquid P1 medium in the dark at 100 rpm at 24 ± 1ºC over a period of 21 days. All values are means ± standard error of three independent experiments (n = 15). Inoculum density ~6 × 10^4 cells/ml suspension.

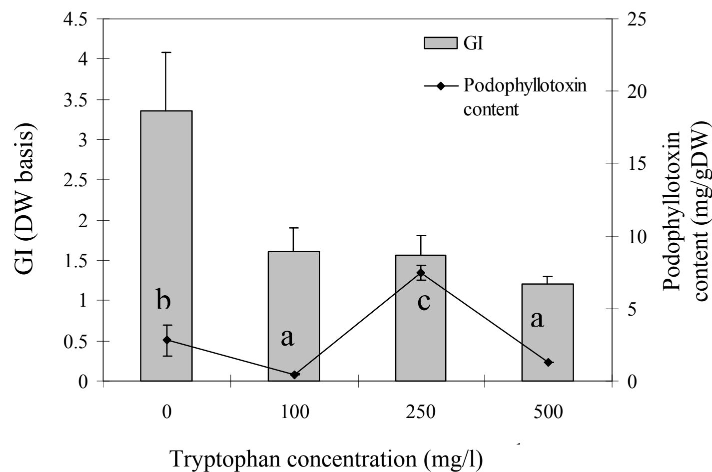

Fig. 2. Effect of tryptophan on biomass yield and podophyllotoxin content in cell suspension cultures of cell line RC4 after a period of 21 days. All values are means ± standard error of three independent experiments (n = 30). Values with different letters are significantly different from each other at p ≤ 0.05 according to ANOVA and DMRT.

Exogenous supply of a biosynthetic precursor to the culture medium is an interesting alternative to increase the yield of a desired product; bottlenecks and flux imbalances in biosynthetic pathways are overcome by supplying exogenous compounds that are relatively close to the desired product (Lin et al. 2003).

Attempts to increase the production of plant secondary metabolites by supplying biosynthetic precursors have been effective in many cases (Fett-Neto et al. 1994, Ghosh et al. 2002, Masoumian et al. 2011, Riedel et al. 2012). Podophyllotoxin, a lignan, is a product of the phenylpropanoid pathway. The biosynthesis of lignans originates from phenylalanine and tyrosine (Ward 1982, Vardapetyan et al. 2003) which are converted via a series of intermediates to coniferyl alcohol, a key precursor in the pathway (Jackson and Dewick 1984b). In the present study, tryptophan, although not a compound from the phenylpropanoid pathway, could improve the accumulation of podohyllotoxin in cell suspension cultures. Tryptophan and phenylalanine/tyrosine are generated from the common precursor chorismate (Maeda and Dudareva 2012), which in turn is produced by the condensation of shikimate with a molecule of phosphoenolpyruvate (Yao et al. 1995). Enzyme chorismate mutase converts chorismate to prephenate which is the first committed step in the synthesis of phenylalaine and tyrosine (Schnappauf et al. 1998). Evidence shows that this enzyme is strongly activated by tryptophan (Schmidheini et al. 1989, 1990, Braus 1991). Thus improvement in podophyllotoxin accumulation in cell suspension cultures might be due to positive feedback activation of phenylalanine and tyrosine biosynthesis by tryptophan (Bentley 1990). Tryptophan can thus been considered as an indirect precursor in the biosynthesis of podophyllotoxin. There is no previous report on the effect of this indirect precursor of lignan biosynthesis on cell suspension cultures of *P. hexandrum*. However, tryptophan adversely affected cell growth. Thus, optimization of a two-stage-culture system for biomass accumulation and podophyllotoxin production might further improve the yield of podophyllotoxin in cell suspension cultures of *P. hexandrum.*

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