

***In vitro* studies on Egyptian *Catharanthus roseus* (L.) G. Don. IV: Manipulation of Some Amino Acids as Precursors for Enhanced of Indole Alkaloids Production in Suspension Cultures**

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Abstract: Egyptian *Catharanthus roseus* (L.) plants produce some pharmaceutical important indole alkaloids, of which the bis and mono-indole alkaloids vinblastine and vincristine are antineoplastic medicines, and ajmalicine and serpentine are antihypertension drugs. Suspension cultures have been induced from leaf explants of *C. roseus* on MS-medium containing 1 mg/l kin under light condition. The influence of L-tryptophane; L-glutamine; L-asparagine; L-cystine and L-arginine at the concentrations of 0; 100; 300 or 500 mg/l on enhanced either cell growth characteristics and indole alkaloids production was investigated. The highest value of mass cell cultures and indole alkaloids production were achieved with modified MS medium containing 300 mg/l of either L-glutamine for mass cell induction or L-tryptophane for enhancement and enrichment of total indole alkaloids; vinblastine and vincristine as compared with other used amino acids at different concentrations.

Key words: *Catharanthus roseus*, suspension cultures, amino acids, total alkaloids, vinblastine and vincristine

INTRODUCTION

Plants are known for the production of a large array of natural products, also referred to as secondary metabolites. Plant secondary metabolites a huge number of natural compounds with a wide diversity in chemical structure. They are economically important to man due to their multiple applications, such as pharmaceuticals, flavors, fragrances, insecticides, dyes, food additives, toxins, etc. However, it is well known that their production is frequently low and depends on the physiological and developmental stage of the plant. The majority of pharmaceutically important secondary metabolites are obtained from wild or cultivated plants, and although some attempts have been made, their chemical synthesis in most cases has not been economically feasible. Therefore, production of plant secondary metabolites by cultivation of plants and chemical synthesis are important agronomic and industrial objectives. As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow-growing plants or difficult to be synthesized with chemical methods (Zaho and Verpoorte, 2007; Zarate and Verpoorte, 2007).

The genus *Catharanthus* (and in particular *C. roseus* L. Don) is well reported for producing biologically active terpenoid indole alkaloids (TIAs) with over 130 compounds isolated and identified (Verpoorte *et al.* 1997; Samuelsson 1999).

Catharanthus roseus (L.) Don. is one of the important medicinal plants especially in Egypt. Furthermore, it is one of the most extensively investigated medicinal plants, chiefly due to the presence of two of the most important anti-tumour agents employed in medicine, the bisindole alkaloids vinblastine and vincristine, which get accumulated in leaves; as well as, other important TIAs used in the treatment of circulatory ailments (anti-hypertensives), such as ajmalicine and serpentine, accumulating in roots (Creasey, 1994). It is known that the

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biosynthesis of vinblastine in *C. roseus* plants begins with the amino acid tryptophan and the monoterpene geraniol, and requires the involvement of at least 35 intermediates, 30 enzymes, 30 biosynthetic and 2 regulatory genes, as well as 7 intra and intercellular compartments (van der Heijden *et al.*, 2004). *C. roseus*, plant became one of major field of interest in modern plant cell biotechnology (Moreno *et al.*, 1994 and Van Der Heijden *et al.*, 1989). Moreno *et al.*, (1994) studied the effect of feeding different terpenoid precursors on alkaloid production. The addition of secologanin and its precursors loganin and loganic acid increased the accumulation of ajmalicine and strictosidine. The highest alkaloid accumulation was observed with loganin feeding. The effect might be due to a higher chemical stability of loganin. On other hand, Morgan and Shanks (2000) studied the effect of the addition of terpenoid and tryptophan branches to the culture medium of *C. roseus* (L.) Don. on metabolic flux to indole alkaloids. They reported that, the feeding of tryptophan at 17 days of the culture cycle produced auxin like effects. Addition of low levels of auxin or tryptophan resulted in significant increase in flux to the indole alkaloids. In this respect, Meijer *et al.* (1993) reported that over twenty enzymes steps are involved in the biosynthesis of terpenoid indole alkaloids (TIAs) in *C. roseus*. These enzymes take place in at least three subcellular compartments, the cytosol, the plastids, and the vacuole. The conversion of L-tryptophan into tryptamine is catalysed by the enzyme tryptophan decarboxylase (TDC). This enzyme is regarded as a putative site for regulatory control of alkaloid biosynthesis and operates at the interface between primary and secondary metabolism. The complete coding region of tryptophan decarboxylase from *C. roseus* was reported by De Luca *et al.* (1989) as the cDNA sequence.

This study presents the effect of L-tryptophane; L-glutamine; L-asparagine; L-cystine and L-arginine at the concentrations of 100; 300 or 500 mg/l on enhancement of indole alkaloids production from suspension cultures of Egyptian *C. roseus*.

MATERIALS AND METHODS

Plant Materials:

Seeds of *Catharanthus roseus* (L.) Don. were kindly obtained from Institute of Horticulture Research, Agricultural Research Centre, Giza, Egypt.

Calli cultures were obtained from leaf explants of *C. roseus* according to the described method by Taha *et al.* (2008).

Suspension Cultures Production:

Suspension cultures were induced from the friable leaf calli cultures (Fig. 1) according to the described method by Torres (1988). The obtained cells were maintained in an agitated liquid MS-medium containing 1 mg/l kin and incubated under day light condition 16/8 at 114 rpm.

Effect of Some Amino Acids on Enhanced of Cell Growth Parameters and Production of Total Alkaloid, vincristine and Vinblastine:

Five amino acids L-tryptophane; L-glutamine; L-asparagine; L-cystine and L-arginine at the concentrations of 0; 100; 300 or 500 mg/l on enhanced either cell growth parameters or relative indole alkaloids production from leaf cell cultures were investigated.

Measurement of Cell Growth Parameters:

The following cell growth parameters were measured at the end of the 14th day of cultivation as follow:

- Cell number : The cell number was calculated according to Neumann (1966).
- Packed cell volume (P.C.V) was determined according to Patrick, (1984).
- Fresh weight (g/ flask).
- Dry weight (g/ flask).

Determination of Total Indole Alkaloids:

Preparation of *in vivo* and *in vitro* derived tissue samples, and determination of total indole alkaloids

were carried out according to the method described by Arvind *et al.* (2007). The obtained total alkaloids of these different calli cultures and *in vivo* derived samples were subjected to HPLC analysis using the following conditions:

Instrument:

- HPLC (waters).
- 600 E delivery system (pump).

Detector:

- 486 UV Detector (Waters associates).

Column:

- Nova Pak C₁₈ (Waters) 3.9 x 150 mm
- These results were integrated by Millennium 32 chromatography.

The standard curves were calculated at wave lengths 254 nm and 280 nm for vincristine and vinblastine, respectively. The percentage of relative total alkaloids; vinblastine (VB) and vincristine (VC) in lyophilized suspension samples to *C. roseus* intact plant were determined and calculated using standard curves.

Statistical Analysis:

All experiments were designed in a completely randomized design and obtained data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

Results:

Maintenance of the Obtained Leaf Cell Cultures:

The obtained leaf suspension cultures were maintained freshly by subculturing every twelve days on newly agitated liquid MS medium supplemented with 1 mg/l kin. Data tabulated in Table (1) shows the effect of different amino acids i.e., L-tryptophane; L-glutamine; L-asparagine; L-cystine and L-arginine at different concentrations on enhancement of various leaf cell growth parameters i.e. cell number ($\times 10^5$), fresh and dry weights (g/flask) and P.C.V. The descending order of maximum cell number ($\times 10^5$) 5.2, 4.82, 4.75, 3.77, 3.65 were recorded with L-glutamine, L-asparagine, L-cystine, L-tryptophane; and L-arginine amino acids, respectively. Furthermore, the highest values of fresh and dry weights (g/flask) 2.93, 2.74, 2.65, 2.43 and 2.35 for fresh weight, and 0.21, 0.19, 0.17, 0.16, and 0.14 for dry weight were recorded with L-glutamine, L-asparagine, L-cystine, L-tryptophane; and L-arginine amino acids, respectively. Also, the highest percentage of leaf P.C.V. 1.62, 1.49, 1.42, 1.39 and 1.35 were recorded with previous amino acids, respectively. The promising and favorable concentration among the different amino acids concentrations was 300 mg/l as compared with 500 and 100 mg/l respectively.

In conclusion, the favorable amino acid for enhancement of various cell growth parameters was L-glutamine at the concentration of 300 mg/l as compared with other used amino acids at different concentrations.

Percentage of Vinblastine, Vincristine and Total Alkaloids Production:

Data illustrated in Fig (2) shows the effect of different amino acids i.e., L-tryptophane, L-glutamine, L-asparagine, L-cystine and L-arginin at different concentrations 0,100,300 and 500 mg/l on enhancement the accumulation rate of total alkaloids, vinblastine and vincristine. The highest relative percentages of total alkaloid 1.57,1.45, 1.32, 1.17 and 1.07 fold as compared with those in *C. roseus* intact plant (1.002 %) were recorded with L-tryptophane, L-glutamine, L-cystine L-asparagine, and L-arginin, respectively. The favorable

concentration of amino acid added to culture medium was 300 mg/l as compared with 500 or 100 or 0 mg/l, respectively.

Also, the highest accumulation percentages of either vinblastine 0.0583, 0.056, 0.0521, 0.0437 and 0.0227, and vincristine 0.0425, 0.0365, 0.0341, 0.0305 and 0.0159 were recorded with L-tryptophane, L-glutamine, L-cystine L-asparagine, and L-arginin, respectively. The suitable concentration of amino acid added to culture

Table 1: Effect of addition of L-tryptophane; L-glutamine; L-asparagine; L-cystine and L-arginine at the concentrations of 0; 100; 300 or 500 mg/l to MS-medium supplemented with 1mg/l Kin on enhancement of *C. roseus* leaf cell growth parameters.

Amino acid concentrations (mg/l)	Cell growth parameters	Amino acids				
		L-tryptophane	L-glutamine	L-asparagine	L-cystine	L-arginine
0.0	Cell number $\times 10^5$	3.2 \pm 0.2	3.2 \pm 0.2	3.2 \pm 0.2	3.2 \pm 0.2	3.2 \pm 0.2
	Fresh weight (g/flask)	1.8 \pm 0.03	1.8 \pm 0.03	1.8 \pm 0.03	1.8 \pm 0.03	1.8 \pm 0.03
	Dry weight (g/ flask)	0.09 \pm 0.005	0.09 \pm 0.005	0.09 \pm 0.005	0.09 \pm 0.005	0.09 \pm 0.005
	P.C.V (%)	0.9 \pm 0.02	0.9 \pm 0.02	0.9 \pm 0.02	0.9 \pm 0.02	0.9 \pm 0.02
100	Cell number $\times 10^5$	3.29 \pm 0.64	4.6 \pm 0.73	4.12 \pm 0.75	4.45 \pm 0.83	3.24 \pm 0.53
	Fresh weight (g/flask)	2.1 \pm 0.4	2.76 \pm 0.3	2.65 \pm 0.43	2.83 \pm 0.35	2.05 \pm 0.35
	Dry weight (g/ flask)	0.12 \pm 0.003	0.15 \pm 0.004	0.13 \pm 0.006	0.12 \pm 0.005	0.10 \pm 0.004
	P.C.V (%)	1.13 \pm 0.02	1.4 \pm 0.05	1.35 \pm 0.06	1.29 \pm 0.09	1.1 \pm 0.03
300	Cell number $\times 10^5$	3.77 \pm 0.43	5.2 \pm 0.32	4.82 \pm 0.45	4.75 \pm 0.53	3.65 \pm 0.33
	Fresh weight (g/flask)	2.43 \pm 0.25	2.93 \pm 0.22	2.74 \pm 0.42	2.65 \pm 0.36	2.35 \pm 0.31
	Dry weight (g/ flask)	0.16 \pm 0.003	0.21 \pm 0.005	0.19 \pm 0.007	0.17 \pm 0.009	0.14 \pm 0.003
	P.C.V (%)	1.39 \pm 0.05	1.62 \pm 0.07	1.49 \pm 0.06	1.42 \pm 0.05	1.35 \pm 0.08
500	Cell number $\times 10^5$	3.54 \pm 0.45	4.8 \pm 0.42	4.73 \pm 0.32	4.62 \pm 0.44	3.49 \pm 0.51
	Fresh weight (g/flask)	2.36 \pm 0.51	2.77 \pm 0.36	2.75 \pm 0.33	2.66 \pm 0.35	2.29 \pm 0.32
	Dry weight (g/ flask)	0.13 \pm 0.007	0.18 \pm 0.006	0.14 \pm 0.005	0.13 \pm 0.005	0.12 \pm 0.005
	P.C.V (%)	1.35 \pm 0.02	1.49 \pm 0.03	1.36 \pm 0.02	1.22 \pm 0.09	1.32 \pm 0.04

Where: Each value is the average of 5 replicates \pm SE and SE= Standard Error

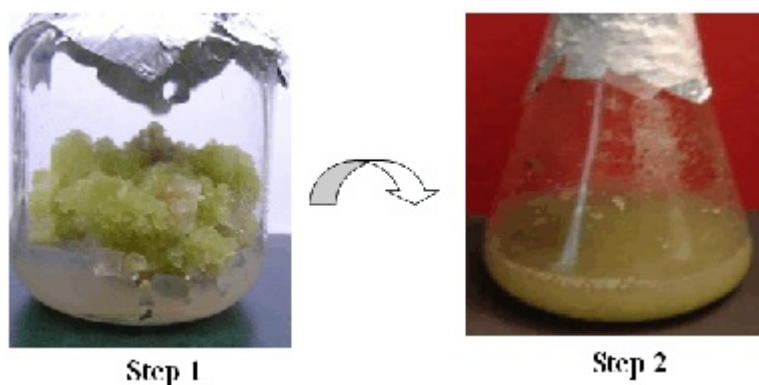


Fig. 1: Steps of Egyptian *C. roseus* suspension production, Step 1 leaf calli cultures, Step 2 suspension cultures after 12 days from inoculation in agitated of MS-liquid medium containing 1 mg/l kin.

medium for achievement of either vinblastine or vincristine was 300 mg/l as compared with 500, 100 and 0.0 mg/l, respectively. Furthermore, the relative percentages of vinblastine and vincristine accumulation as compared with those in intact plant were 1.24, 1.19, 1.11, 0.92 and 0.48 fold for vinblastine in intact plant (0.047%). However they were 1.25, 1.07, 1.01, 0.89 and 0.47 fold for vincristine in intact plant (0.0339 %).

Furtherer, it can be concluded that the addition of either L-glutamine and L-tryptophane as amino acids at the concentration of 300 mg/l to MS- culture medium enhanced of both cell growth parameters and

enrichment the accumulation rates of either total alkaloids, vinblastine and vincristine in leaf of Egyptian *C. roseus* suspension cultures.

Discussion:

Catharanthus roseus (L.) Don. alkaloids, especially vincristine and vinblastine are two of the most important plant-derived drugs as anticancer throughout the world. Balandrin and Klocke (1988) mentioned that about 500 kg of leaves are needed to produce just one gram of purified vincristine. This means that 12-15 tons are required to produce one ounce of drug. This situation reflects the importance of the present investigation.

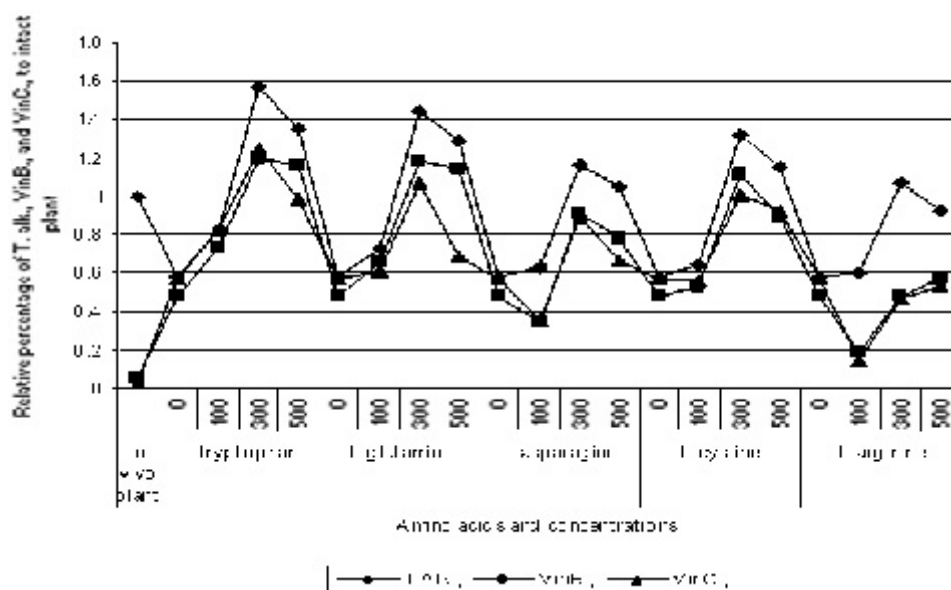


Fig. 2: Effect of addition of L-tryptophane; L-glutamine; L-asparagine; L-cysteine and L-arginine at the concentrations of 0; 100; 300 or 500 mg/l to MS-medium supplemented with 1mg/l Kin on the relative percentage of enhancement the accumulation rate of total alkaloids, vinblastine and vincristine in leaf of *C. roseus* suspension cultures as compared with those in intact plant.

In recent years, considerable interest has been shown in the production of secondary metabolites such as pharmaceuticals from plant cells. Over 25 % of all prescription medicines are still derived from plants (Ganapathy and Kargi, 1990). So, it may become critical to develop alternative sources of important natural products. Plant cell and tissue cultures represent a promising and an unique source for valuable phytochemicals such as flavors, fragrances, and pharmaceuticals (Jacqueline *et al.*, 1999). Furthermore, cell suspension cultures could be used for the large scale culturing of plant cells from which active agents can be extracted and prepared. The principle advantage of this technology is that it ultimately provides a continuous and reliable source of active agent's year around. In addition, compounds from tissue cultures are more easily purified because of the simple extraction processes used and also the absence of contaminants such as pigments, thus consequently, reducing the production and processing costs (Bourgand *et al.*, 2001). However, the amount produced as well as the rate of production of useful metabolites in plant cell cultures is still very low. To overcome these problems, some methods for inducing the release of useful products into the culture medium have been tested. These include pH cycling (Renaudin, 1981), use of permeabilizing agents (Brodelius and Nilsson, 1983), high ionic strength (Tanaka *et al.*, 1985), and electroporation (Brodelius, 1988). Plant tissue culture techniques have been studied extensively for the production of the anti-cancer compounds, vincristine and vinblastine from cell of *Catharanthus roseus* Constabel *et al.* (1981). On other hand, Namdeo, *et al.* (2007) reported that the key to successful protocol using precursor feeding to plant cell

culture system lies in identification of cheapest by product of the other process which can be converted to desired secondary metabolites by selected plant cell line. Furthermore, in agreement of our obtained results, Junaid *et al.* (2008) reported that the highest levels of *C. roseus* embryogenesis, proliferation and maturation were observed for moderate concentrations of L-glutamine followed by L-asparagine. Also, Marzena and Henryk (2006) studied the effect of precursors amino acids (phenylalanine and cysteine) on glucotropaeolin production in hairy root cultures of *Tropaeolum majus* L. They reported that glucotropaeolin content and yield were 2 fold enhanced after treatment with precursors amino acids. Moreover, Jie *et al.* (2005) reported that the production of phenylethanoid glycosides was enhanced by feeding precursors L- phenylalanine at 0.2 mmol l⁻¹ to cell culture of *Cistanche deserticola*. The feeding cell line was 75% higher than that obtained in the cell cultures without precursors. Further, in conformity with our obtained results, Serap *et al.* (2002) reported that supplying tryptamine or tryptophan along with the iridoid precursors to transgenic cell lines S1 of *C. roseus* resulted in even further increase of alkaloid accumulation. On other hand, Morgan and Shanks (2000) studied the effect of the addition of terpenoid and tryptophan branches to the culture medium of *C. roseus* (L) Don. on metabolic flux to indole alkaloids. They reported that, the feeding of tryptophan at 17 days of the culture cycle produced auxin-like effects. Addition of low levels of auxin or tryptophan resulted in significant increase in flux to the indole alkaloids. On same respect, the effect of feeding precursors (tryptamine and loganin) of transgenic cell line of *C. roseus* under conditions of high-STR (strictosidine syntheses) on alkaloids accumulation was investigated by Serap *et al.* (1998). They reported that, the utilization of tryptamine for alkaloids biosynthesis enhanced metabolic flux through the indole pathway.

ACKNOWLEDGEMENT

This research was carried out under the program of the "National Strategy for Genetic Engineering and Biotechnology" this program is financed and supported through Ministry of State for Scientific Research and is administered by Egyptian Academy of Scientific Research and Technology.

Abbreviations:

MS : Murashige and Skoog medium.
Kin : Kinetin, 6-furfurylaminopurine.
(VB) : vinblastine.
(VC) : vincristine.

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