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In Vitro Studies on Egyptian *Catharanthus roseus* (L.) G.Don V: Impact of Stirred Reactor Physical Factors on Achievement of Cells Proliferation and Vincristine and Vinblastine Accumulation.

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ABSTRACT

Catharanthus roseus (L.) G. Don, the Madagascar periwinkle, synthesizes numerous terpenoid indole alkaloids (TIAs) used in medicine such as vinblastine and vincristine . *C. roseus* cell cultures were developed from shake-flasks to scale up through bioreactor. In this study the physical conditions of stirred tank bioreactor (STB) such as controlled or uncontrolled pH and different aeration values rates (0.5; 0.8 and 1 l/min) affecting on increasing of cell suspension production and relative percentage of vincristine and vinblastine accumulation were investigated. Results indicated that using uncontrolled pH medium produced 615.35 and 50.76 (g/run) of cells fresh and dry weights, respectively. As well as resulted 1.38 (% DW); 5.61 and 14.52 (fold) of total alkaloids ; vincristine and vinblastine, respectively , as compared with intact plant of *C. roseus* after 15 days of cultivation. However, this treatment produced (10.74) fold of vincristine compared with intact plant. Concerning aeration levels it was found that the capacity of 0.8 l/min was the best as aeration level with uncontrolled pH medium which produced 734.12, 67.91 (g/run) of cells fresh and dry weights , respectively. However, it produced 1.4 (% DW); 13.47 and 7.94 (fold) compared with intact plant for total alkaloids, vincristine and vinblastine, respectively.

Key words: *Catharanthus roseus*; cell suspension; bioreactor; vincristine and vinblastine.

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INTRODUCTION

Medicinal plants are inexhaustible source of life saving drugs for majority of the world's population. *Catharanthus roseus* (L.) G. Don. (Apocynaceae), is one of the important medicinal plants and especially in Egypt. In addition, it is one of the most extensively investigated medicinal plants (Taha *et al*., 2009). Traditionally, different parts of it used in the treatments of various diseases (*viz.* diabetes, menstrual regulators, hypertension, cancer etc.). Moreover, more than 130 alkaloids have been isolated from different parts; amongst which two important alkaloids (Vinblastine and Vincristine used in cancer treatment) present in very low concentrations (Junaid *et al*., 2010).

Culturing of plant cells, tissues or organs for the production of pharmaceutically valuable compounds of commercial interest has gained popularity over the last few years (Canter *et al*., 2005and Murthy *et al*., 2008). The evolving demand of secondary metabolites in recent years resulted in a great interest, in secondary metabolism, especially in the possibility to alter the production of bioactive molecules by means of cell culture technology (Vijaya *et al*., 2010). In vitro aseptic suspension culture of plant cells, tissues or organs under controlled environmental conditions has been developed over the past 50 years, primarily for the production of valuable medicinal secondary metabolites such as shikonin and paclitaxel (Hellwig *et al*., 2004). Advancement of biotechnological research has made plant cell suspension cultures capable of producing particular medicinal compounds at a rate similar or superior to that of naturally grown whole plants. However, to fulfill the demand of the increasing population, the major challenge is how to adopt those technologies under laboratory conditions for large scale production of bioactive molecules from plant cells that are reproducible, safe, and economically viable. Further, application of bioreactor technology is the key step toward commercial production of bioactive molecules by plant biotechnology. Compared to naturally grown “whole wild plants” or traditionally grown “whole transgenic plants” their production in bioreactors ensures defined control process conditions. Thus, minimizes or even prevents variations in yield and quality of the products, which simplifies process validation and product registration (Sivakumar, 2006 and Eibl and Eibl, 2008). Moreover, proper engineering of bioreactor significantly affects cultivation results by accomplishing and controlling the optimal environment for effective cell growth and production of secondary metabolites. Moreover, the important process parameters inside the bioreactor vessel such as pH, temperature, dissolved oxygen and carbon dioxide evolution rate can be monitored and controlled (Sivakumar, 2006). Indeed, to design an appropriate bioreactor system for a particular bioprocess, intensive research efforts on the biological system, such as cell growth, metabolism, genetic manipulation, and other product formation are needed to understand about the physical and chemical environments required for the efficient cultivation of plant cells or roots (Zhong, 2010).

Culturing of plant cell in bioreactors have advantages over whole plants for sustained biopharmaceuticals production including (1) simplified purification particularly for products secreted into the extracellular medium ; (2) consistency in product quality and homogeneity achievable under controlled environmental conditions; (3) ease of compliance with cGMP requirements; (4) elimination of the need for cultivation and manipulation of greenhouse or field grown plants which can be quite labor intensive; (5) ability to use inducible promoter systems; (6) reduced potential for endotoxin and mycotoxin contamination derived from the

plant and soil source, and (7) minimal cell-to-cell communication resulting in the possible reduction of systemic post-transcriptional gene silencing (PTGS), which occurs *via* plasmodesmata as well as the vascular system in whole plantsv(Crawford and Zambryski 1999).

Stirred-tank bioreactors (STB) equipped with suitable impellers can provide high volumetric mass transfer coefficients and a homogeneous environment enabling suspended plant cell growth production to be controlled consistently (Kieran 2001; Zhong *et al*., 2002).

This study is the first record in the field of controlling of stirred reactor (STB) physical factors such as controlled or uncontrolled pH medium and different aeration values affecting on *in vitro* cell suspension increasing value and relative percentage of vincristine and vinblastine accumulation as a source of antineoplastic agent's from Egyptian *Catharanthus roseus* (L.) plant.

MATERIALS AND METHODS

Plant Materials

Source of *C. roseus* seeds

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

Callus induction

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

Suspension cultures initiation

Suspension cultures were induced from the friable leaf calli cultures according to 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 26 º C and 120 rpm according to extracted results by Taha *et al*.(2009).

Bioreactor studies

In this experiment 2-L turbine stirred tank bioreactor (STB) was used with a working volume of 1.5-1.7 L (B. Braun, Biotech, International, Germany). The culture was aerated through a sintered steel sparger. The flow rate was set up according to the type of experiment and maintained at the normal level with a mass flow control system until the end of the culture period. Six bladed turbine impellers (D = 45 mm) were used for mixing; rotation speed was 120 round /min (rpm). The temperature was maintained at 26 °C with thermostatic outlet spongy sheet rounded the vessel. Aeration was performed by filtered sterile air at the rate of 0.5 l/min Dissolved oxygen concentrations were measured with sterilizeable oxygen electrode (Ingold). Dissolved oxygen concentration was monitored with

a sterilizeable pO_2 electrode. For maintaining different levels of dissolved oxygen concentrations in the bioreactor broth, volume of the inlet air was dosed by a mass flow controller connected with software and pO_2 electrode. The formation of foam was prevented by adding a silicon-based antifoaming agent (1% w/w) at a regular time intervals. The bioreactor was inoculated with one part suspension culture and five parts of medium, and the cell cultures were kept at 26 ±1°C. The MS-nutrient medium containing cell lines were provided into glass tank bioreactor under sterilized air condition. At the end of fifteen days after inoculation with *C. roseus* leaf cell culture, obtained cells were harvested and chemically analyzed for vinblastine and vincristine concentration. The relative percentages of vincristine and vinblastine were recorded as compared with intact *C. roseus* plant.

The following parameters affecting on either mass cell culture production (fresh and dry weights g/run) or enhancement of target alkaloids production (vinblastine and vincristine) from leaf suspension cultures of *C. roseus* were investigated as follow:

- Effect of uncontrolled or controlled pH medium at the degree of 5.7 using either 0.2 N NaOH or 0.2 N HCL through ADI 1030 Bio-controllers (Applikon) equipped with sterilizeable pH–electrode (Ingold) and peristaltic pumps for alkali or acid addition . The rhythm of lighting system was 16/8 h in light/dark. The light intensity was 1500 Lux from white cooling florescent lamps.

- Effect of different aeration values 0.5, 0.8 and 1.0 l/min.
  Each experiment had been done for 15 days after inoculation Furthermore; the effect previous parameters on enhancement of either cell growth parameters or vincristine and vinblastine accumulation were recorded. At the end of fifteen days of inoculation, the obtained cells were harvested, lyophilized and chemically analyzed for vinblastine and vincristine contents. The percentage of these target compounds were recorded as compared with intact *C. roseus* plant.

Preparation of total alkaloids from *C. roseus*

Determination of total indole alkaloids: Preparation of *in vivo* and *in vitro* derived tissue samples (5 g), of total indole alkaloids were carried out according to the method described by Arvind *et al*. (2007). The obtained total alkaloids of derived samples were subjected to HPLC analysis using the following conditions:

Instrument: HPLC (water,s). 600 E delivery system (pump).

Detector: 486 UV Detector (Water,s associates).

C18 Column: Nova Pak C (Water,s) 3.9 x 150 mm.

The results were integrated by Miliennium 32 chromatography. The standard curves were calculated at wave lengths 254 (nm) and 280 (nm) for vincristine and vinblastine, respectively. The percentage of total alkaloids, vincristine (VC) and vinblastine (VB) in different cell lines as well as intact plant samples were determined and calculated using standard curves.

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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).

Accurralty weighted authentic samples of vinblastine and vincristine were used injected to determine the standard curves. Vincristine was recorded at Rt = 1.177 min. and vinblastine was recorded at Rt = 2.104 min.. The standard curves were calculated at wave lengths 254 nm and 280 nm for vincristine and vinblastine, respectively. Results are shown in Table (1).

Table 1: Standard curve data of vincristine and vinblastine

Vincristine		Vinblastine	
Conc., (µg/ml	Area x 10^{-6}	Conc., (µg/ml)	Area x 10^{-6}
10	0.363	0.025	0.020
20	0.609	0.05	0.039
30	0.957	0.10	0.079
40	1.2550	0.15	0.113
50	1.543	0.20	0.150

RESULTS

Effects of physical factors (controlled, uncontrolled pH and different aeration levels) on cell proliferation (g/run); total alkaloids and relative percentage of vincristine & and vinblastine accumulation in *C. roseus* suspension cultures cultured in STB (2L).

Effect of controlled and uncontrolled of pH medium.

Presents data in Figs. (1 and 2) show that the effect of controlled and uncontrolled pH medium on *in vitro* mass cell production from leaf explants of *C. roseus* during 15 days of cultivation in STB (2L). The pH of modified MS medium adjusted at 5.7 using either 0.2 N NaOH or 0.2 N HCL for controlled pH medium and the other physical conditions were adjusted at rotation speed 120 rpm, temperature 26 ±1ºC;aeration at dissolved oxygen 0.5 L/min). Parameters of physical growth rate were recorded as fresh or dry weights at 0,5,10 and 15 days of inoculation with ~ 100 (g) of fresh cells/ run. Data clearly stated that, the highest mass cell production 615.35 and 50.76 (g/run) were recorded as fresh and dry weights, respectively at the 15 th day of cultivation in STB with uncontrolled pH medium. As well as the highest mass cell production using controlled pH medium at 5.7 produced 518.7 and 48.16 (g/run) for fresh and dry weights, respectively at the 15 th day of cultivation. However, the significant mass cell production (525.27 and 45.35 g/run) were recorded for fresh and dry weights, respectively after the 10 thday of cultivation in STB without controlled of pH medium.

Concerning the effect of controlled or uncontrolled of MS pH medium as a physical factor on enhancement the accumulation rate for total alkaloid as well as vincristine and vinblastine as relative percentage to *C. roseus* intact plant after 15 days of cultivation was

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investigated. Data tabulated in Table (2) demonstrated that the highest percentage of total alkaloids (1.38 and 1.12) were recorded with uncontrolled and controlled pH media, respectively. On the other hand, the highest values of vincristine (10.74 and 5.61 fold) were recorded with controlled and uncontrolled pH medium , respectively, compared with *C. roseus* intact plant. However and in contrast, the highest values of vinblastine (14.52 and 5.44 fold) were recorded with uncontrolled and controlled of pH media, respectively, as compared with *C. roseus* intact plant.

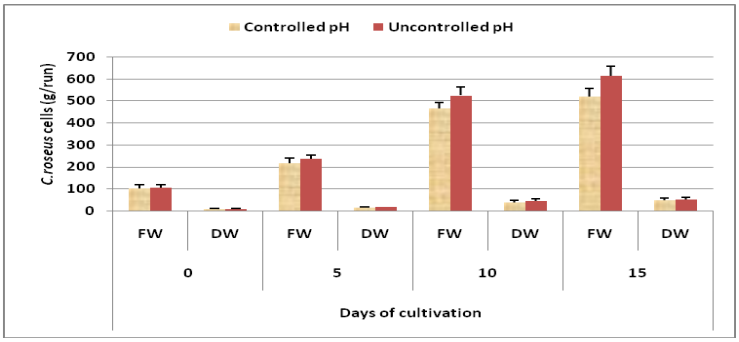

Figure 1: Effect of controlled and uncontrolled pH media on fresh and dry weights (g/run) of mass cells production from leaf explants of *C. roseus* cultured in STB (2L).

Where: pH was 5.7 in controlled MS medium , rotation speed 120 rpm, temperature 26 ±1ºC;aeration at dissolved oxygen 0.5 L/min. Initial inoculum fresh weight was ~100 g cells /run.

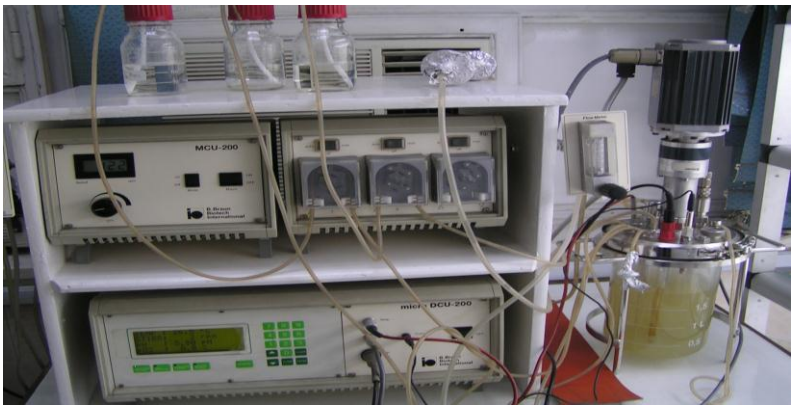

Figure 2: B-Braun Biotechnology International stirred tank bioreactor (2 L).

Table (2). Effect of controlled and uncontrolled of pH media on total alkaloids and relative percentage of vincristine (vinc.) and vinblastine (vinb.) of *C. roseus* leaf cell cultures, cultured in STB (2L).

Type of plant materials	Total alkaloids (% DW)		Relative % Vinc.		Relative % Vinb.	
	Con	Uncon.	Con.	Uncon.	Con.	Uncon
Leaf suspension	1.12	1.38	10.74	5.61	5.44	14.52
Intact plant	1.01	1.01	1	1	1	1

Where pH of cultured medium was adjusted to 5.7, rotation speed 120 rpm, temperature 26 ±1ºC;aeration at dissolved oxygen 0.5 L/min. Light rhythm was 16/8 h from cool white inflorescence lamp.

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Effect of different aeration values (0.5, 0.8 and 1.0 l/min).

Regarding the effect of aeration rates (0.5, 0.8 and 1 l/ min) on achievement of cell growth rate during the 15 days of cultivation in STB. Data presents in Fig. (3) clearly show that the maximum values 734.12 and 67.91 (g/run) were recorded at the 15 th day of cultivation with the aeration rate of 0.8 l/ min for cells fresh and dry weights, respectively. As well as, the significant cell growth rates as fresh or dry weights (459.76 and 37.86 g/run) were recorded at the 10 th day of cultivation with the aeration rate of 0.8 l/min. However, using aeration rate at the level of 0.5 l/min produced the lowest either cells fresh or dry weights (585.77 and 45.21 g/run) compared with the other aeration rates (1or 0.8 l/min), respectively. On the other hand, the effect of those aeration systems on enhancement the accumulation percentage of total alkaloids and relative percentage of vincristine & vinblastine in *C. roseus* cell cultures were investigated. As shown in Table (3) the descending order of the maximum percentage of total alkaloids 1.40, 1.22 and 1.16 (% DW) were recorded with the aeration rate of 0.8, 1 and 0.5 (l/min), respectively. Moreover, the highest relative percentages 13.47 and 7.94 (fold) were recorded for vincristine and vinblastine accumulation rates, respectively, with the aeration rate of 0.8 l/min. as compared with intact plant with uncontrolled of pH medium.

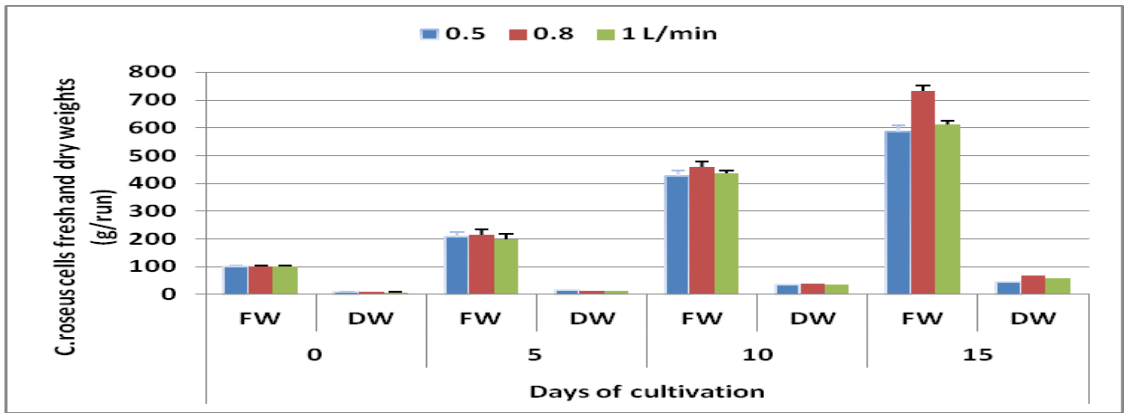

Figure 3: Effect of different aeration rates on fresh and dry weights (g/run) of mass cells production from leaf explants of *C. roseus*,cultured in STB (2L).

Where: pH was in uncontrolled state, rotation speed 120 rpm, temperature 26 ±1ºC;Initial inoculum fresh weight was ~100 g cells /run.

Table 3: Effect of different aeration rates (0.5,0.8 and 1 l/min) on total alkaloids and relative percentage of vincristine (vinc.) and vinblastine (vinb.) of *C. roseus* leaf cell cultures, cultured in STB (2L).

Aeration levels (l/min)	Total alkaloids (% Dw)	Relative (%) Vinc.	Relative (%) Vinb.
0.5	1.16	10.95	5.68
0.8	1.40	13.47	7.94
1.0	1.22	11.86	5.83
Intact plant	1.01	1	1

Where is the pH was in uncontrolled state, rotation speed 120 rpm, temperature 26 ±1ºC; light rhythm was 16/8 h from cool white inflorescence lamp.

DISCUSSION

Higher plants are recognized as important sources of a wide range of biochemicals, used as drugs, pesticides, flavourings and fragrances (Thorpe, 1990, 2007 and Stasolla & Thorpe 2011). In addition, plant cell culture provides an alternative approach, which may be attractive under certain circumstances: if, for example, the source plant is difficult to cultivate, has a long cultivation period or has a low metabolite yield; if chemical synthesis has not been achieved or if it is technically problematic. Metabolite yield by the cell culture may significantly exceed that observed in the intact plant. Thus, using this technology, the metabolite can be produced under controlled and reproducible conditions, independent of geographical and climatic factors (Kieran *et al*., 1997).

Plant bioreactors are attractive expression systems for economic production of pharmaceuticals. Various plant expression systems or platforms have been tested with certain degrees of success over the past years. However, further development and improvement are needed for more effective plant bioreactors (Yansong *et al*., 2008).

This study is the first record concerning the effect of some physical factors affecting enhancement of cell suspension proliferation and relative percentage of vincristine and vinblastine production from leaf explants of *C. roseus* cell cultures that cultured in serried reactor.

Concerning the effect of controlled or uncontrolled pH degree on achievement of cells growth; total alkaloid and relative percentage of vinblastine and vincristine accumulation. Results indicated that using uncontrolled of pH medium produced 615.35 and 50.76 (g/run) for cells fresh and dry weights, respectively. Moreover, recorded 1.38 (% DW) of total alkaloids ; 5.61 and 14.52 (fold) as compared with intact plant of *C. roseus* for vincristine and vinblastine , respectively, after 15 days of cultivation in serried reactor. However, it produced 10.74 fold of vincristine compared with intact plant. In this regard de Touchet *et al.* (1991) reported that embryogenic oil palm cells grow successfully in a stirred-tank bioreactor with an increase in biomass of 3.5-fold per month. This result agrees with the data reported by for oil palm flask cultures,as well as for other monocotyledons such as wheat (Redway *et al*., 1990) and maize (Emons and Kieft, 1995) .

Regarding the effect of different aeration systems on enhancement of both cells proliferation as well as vinblastine and vincristine accumulation , it was found that the capacity of 0.8 l/min was the best as aeration level with uncontrolled pH medium which produced 734.12, 67.91 (g/run) of cells fresh and dry weights , respectively. However, this treatment produced 1.4 (% DW) ; 13.47 and 7.94 (fold) as compared with the intact plant for the accumulation of total alkaloids as well as vincristine and vinblastine , respectively. In this regard and in agreement with the obtained results, Dae-Sung *et al*.(2008) reported that 43% DO, two of the agitation rate (120 and 225 rpm) resulted in more than 200 % increase in biomass of oil palm suspension culture compared to the initial inoculum. Moreover, they reported that excessive agitation (335 rpm) was correlated with poor

growth. Moreover; and in close of obtained results; Schlatmann *et al*. (1995) reported that oxygen requirements of plant cells are relatively low for cell growth, but may significantly increase during metabolite synthesis. On the other hand, Mansouri *et al*.(2005) reported that total phenolic content of kharak date palm fruits(DPF) varied from 2.89 to 141.35 mg gallic acid equivalents (GAE)/100 g dw sample . However, Al-Farsi *et al*. (2007) reported that total phenolic content of date palm varied between 172 and 246 mg gallic acid equivalents/100 g fresh weight of date palm.

CONCLUSIONS

The obtained results clearly showed that the problems of scaling-up and semi-industrial production of secondary products at commercial level through bioreactor could be overcome by optimization of the operating conditions, including chemical and physical factors.

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