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ESTIMATION OF COLCHICINE IN CELL SUSPENSION CULTURES OF *Gloriosa superba* L.

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ABSTRACT : Callus was induced from different explants of *Gloriosa superba* L on MS medium supplemented with different concentrations of 2,4- D. Cell suspension cultures derived from callus of Gloriosa superba L. were established quantified for the colchicine content using High Performance Liquid Chromatography. Effect of time on the growth of cells and colchicine production by cell suspension cultures in liquid medium was also studied. Maximum growth index (0.331 ± 0.0016) was obtained after four weeks of cell growth. Itt was observed that the colchicine yield reached 13.33 ± 0.024 µg/g dry wt. in cells and 59.30 ± 0.54 µg/l in residual liquid medium after four weeks of culture in liquid growth medium and thereafter there was reduction in biomass as well as colchicine content.

Keywords : *Gloriosa superba, in vitro, cell suspension, growth index, colchicine*

Colchicine, an alkaloid, is a natural compound found mostly in plant species belonging to Colchicaceae family. Colchicine has been reported to have a multitude of biological activities, including anti-mitotic, anti-inflammatory, anticancer and antimicrobial effects (Joshil *et al.,* 14). Due to its diverse uses and high demand, colchicine is a valuable product for the pharmaceutical industry (Ade and Rai, 1; Sapra *et al.,* 20). There are few reports on production of colchicine from in vitro cultures of Gloriosa superba L. (Kumar *et al.,* 16; Ghosh *et al.,* 9). The production of colchicine under in vitro conditions was been studied in suspension cells of Colchicum autumnale (Hayashi *et al.,* 11) and Colchicum hierosolymitanum Feib. (Daradekeh *et al.,* 7).

In vitro techniques has been attempted in Gloriosa superba by many workers to increase the yield of colchicine and meet the demands of the pharmaceutical industry (Kannan *et al.,* 15; Jana and Shekhawat, 13; Yadav *et al.,* 23). These demands can be overcome by adopting cell culture techniques which offer an attractive alternative method to produce this compound (Baldi and Dixit, 5). Even the root cultures of Gloriosa superba were used for the production of colchicine (Ghosh *et al.,* 9), while Sivakumar *et al.,* (21) reported production of colchicine from callus cultures.

The present paper reports the production of colchicine from cell suspension cultures of *Gloriosa superba* L. Also, the conditions of the cell growth and synthesis of colchicine with respect to the time period were optimized.

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MATERIALS AND METHODS

Plant material

Tubers of Gloriosa superba collected from Botanical garden, University of Jammu, J&K were surface sterilized as per the sterilizing agents used by Mahajan *et al.* (19). Disinfected tubers were inoculated on basal MS medium (Murashige and Skoog, 18) with 3% sucrose and 0.7% agar. The pH of the medium was adjusted at 5.8 using 0.1 N NaOH before autoclaving at 121°C for 20 min. The cultured tubers were grown at 28°C with 16-h photoperiod under fluorescent light conditions. The sprouted tubers after 4 weeks were transferred to MS medium supplemented with 2.0mg/l 6-BA and 0.5 mg/l kinetin for shoot elongation.

Callus Induction

Expanded leaves from in vitro raised plantlets were cut into pieces and were used as explants for callus induction. The leaves were inoculated on MS medium containing growth regulators (Mahajan *et al.,* 18) with few modifications (Table 1). Induced callus after four weeks was transferred to fresh medium containing 4.0mg/l 2,4-D and the process was repeated every two weeks for multiplication.

Cell suspension cultures

Cell suspension cultures were established after cutting the calli into small pieces and then inoculating them in 100ml conical flask containing 25ml of liquid MS medium supplemented with 4.0mg/l 2,4 D (growth medium). The pH of the medium was adjusted to 5.8. Liquid medium containing the calli pieces were incubated on a rotary shaker (120 rpm) at 28°C under dark and subcultured after every two weeks in fresh

growth medium so as to get a homogeneous cell suspension.

Growth pattern of *Gloriosa* cells in suspension cultures

The growth curve of cell suspension cultures was determined by harvesting the cells periodically (0-5 weeks). The growth index of biomass and colchicine content from these in vitro grown cells as well as growth medium was determined (Li *et al.,* 17). Growth index of biomass was calculated using following formula (He-Ping *et al.,* 12).

$$\text{Growth index} = \frac{(\text{Final fresh weight} - \text{Initial fresh weight})}{\text{Initial fresh weight}}$$

Extraction of Colchicine from cells

Cells were harvested by centrifugation at 3000 rpm for 15 m to remove the medium and then washed with distilled water. The fresh weight of the cells was recorded and then the cells were dried at 25 ± 2°C until constant weight was achieved (Baldi and Dixit, 5). The cells were then extracted using method described by Ali *et al.* (3). Briefly, the dried cells were finely ground using liquid nitrogen. 10 ml HPLC grade methanol was added to finely ground powder. The mixture was sonicated for 10 min; three times with a resting period of 30 min in between and centrifuged at 8,000 rpm for 10 min. The supernatants were collected, filtered through a 0.45 µm membrane filter and stored at 4°C till further use.

Extraction of Colchicine from liquid media

Colchicine content of the residual liquid medium was also analysed. Liquid medium was extracted thrice with chloroform (1:2 v/v) with continuous shaking at room temperature for 2 h. The chloroform extract was separated from aqueous phase by a separating funnel. Finally the chloroform extracts were evaporated to dryness and the residue was dissolved in 10 ml HPLC grade methanol, filtered and subjected to HPLC (Ghosh *et al.,* 8).

HPLC analysis

The high performance chromatographic separations were carried out on Waters HPLC system using a C18 (100 × 3mm) column as per protocol given by Bodoki *et al.* (6). The mobile phase consisted of Acetonitrile : Water (75:25 v/v) and was delivered at a rate of 1mL min-1. The temperature was maintained at 42°C during all experiments. The methanolic standard solutions of colchicine (10µL) were injected and the analytes were detected at 245nm. The retention time for the colchicine was approximately 3.2 min. Identification of colchicine was done by comparing the retention time of the sample with that of the standard colchicine (Sigma Aldrich, USA).

RESULTS AND DISCUSSION

Establishment of shoots and their multiplication

Sprouting was initiated from tuber explants after three weeks of their inoculation on MS basal medium. Transfer of newly emerged shoots to MS with 2.0mg/l 6-BA and 0.5 mg/l Kinetin led to fast shoot proliferation and multiple shoot formation. Venkatachalam *et al.* (22) also reported that the use of BA and Kinetin as growth regulators results in shoot proliferation. The leaves from these in vitro generated plantlets were used as explants for callus induction.

Callus induction

Callus was induced from the in vitro grown leaves on different concentrations of 2,4-D after six weeks of inoculation (Table 1). 2,4-D at the concentration of 3.0mg/l resulted in creamy white and friable callus. Even, the use of growth regulator 6-BA (Benzylamin opurine) along with 2,4-D resulted in callus formation. Arumugam and Gopinath (4) and Gopinath *et al.* (10) also supported the use of different cytokinins along with 2,4-D for callus formation.

Table 1 : Effect of different growth regulators on callus induction

Medium	Number of Explants	Callus induction
MS + 2.0 mg/l 2,4D	60	10.33 ± 0.44
MS + 2.5 mg/l 2,4D	60	18.00 ± 0.50
MS + 3.0 mg/l 2,4D	60	18.77 ± 0.32
MS+ 2.5 mg/l 2,4D +1.0 mg/l 6-BA	60	15.77 ± 0.59
MS+ 3.0 mg/l 2,4D +1.0 mg/l 6-BA	60	18.11 ± 0.48
MS+ 4.0 mg/l 2,4D	60	18.44 ± 0.55

Each experiment consists of three replicates and data is presented as Mean ± Standard Error

HPLC analysis

The HPLC method can be widely used as a standard technique for rapid and accurate qualitative and quantitative determination of colchicine from extracts of in vitro generated cultures of *G. superba* (Fig 1 and 2). This method has a number of advantages over the other analytical procedures such as TLC and spectrophotometry for the quantification of even minute quantity of colchicine in in vitro samples (Alali *et al.,* 2).

Growth Kinetics of Cells Suspension Cultures

The growth index of cells and production of colchicine in the cells and medium is presented in (Table 2). Accumulation of maximum biomass (0.33 ± 0.001) was recorded after 28 days (Table 2, Fig 3). However, the maximum quantity of colchicine in cells and medium after 28 days was 13.33 ± 0.02 and 59.30 ± 0.54 respectively (Table 2, Fig 4). Ghosh *et al.* (8) also observed similar results in root cultures of *Gloriosa superba*.

Table 2 : Effect of Time Course on Growth of Cells and Concentration of Colchicine in Cells and Medium

Number of days	Growth index	Concentration of colchicine in cells (µg/g)	Concentration of colchicine in Medium (µg/l)
0	0	2.84 ± 0.0126	Not detected
7	0.07 ± 0.0001	4.94 ± 0.0172	24.52 ± 0.019
14	0.14 ± 0.0013	6.41 ± 0.25	39.3 ± 0.47
21	0.26 ± 0.011	10.64 ± 0.012	54.78 ± 0.58
28	0.33 ± 0.0016	13.33 ± 0.0243	59.30 ± 0.54
35	0.26 ± 0.0002	11.1 ± 0.10	55.01 ± 0.0045

Each experiment consists of three replicates and data is presented as Mean ± Standard Error

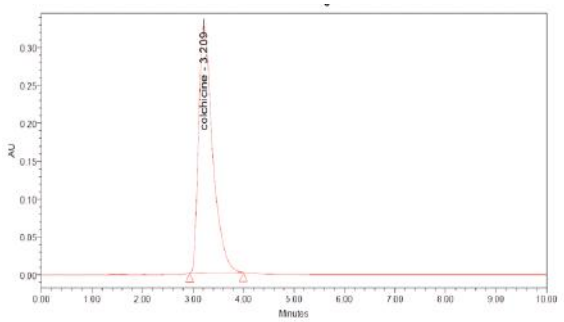

Fig 1: HPLC Chromatogram of Standard Colchicine

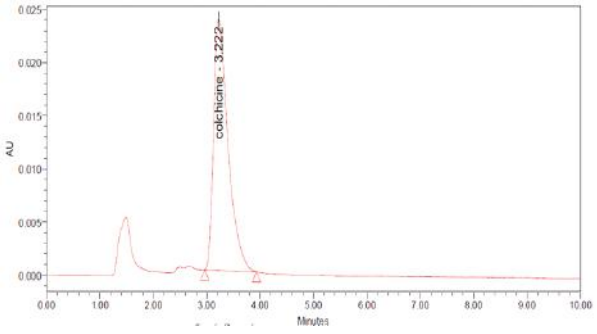

Fig 2: The Chromatogram showing the presence of Colchicine in Cell Culture Extract

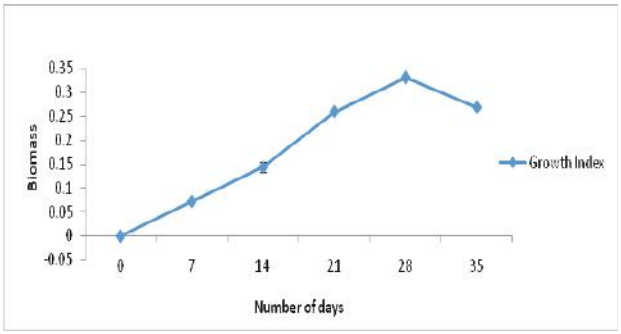

Fig 3: Growth kinetics of cells of *Gloriosa superba* in suspension cultures.

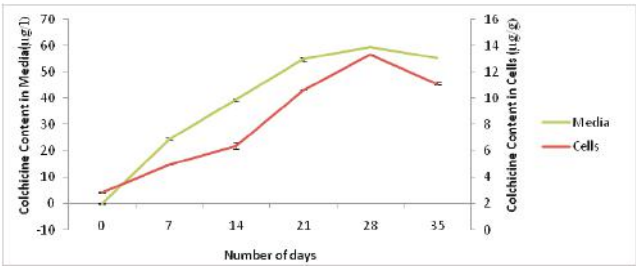

Fig 4: Colchicine Content in Suspension Culture Cells and liquid medium.

CONCLUSIONS

The present study provides a system which is suitable for micropropagation and callus induction from leaf explants of G. superba. Explants could be cultured under darkness on MS medium supplemented with 3mg/l and 4mg/l 2,4-D. Once calli were obtained, they were transferred to liquid medium containing same concentration of 2,4-D for the establishment of cell suspension cultures and analysis of their secondary metabolite, colchicine. Biomass and colchicine production increases with time in Gloriosa superba suspension cultures upto four weeks. However, efforts have been made to improve the yields of colchicine in cell cultures of *G. superba* by adopting various culture techniques such as elicitation and precursor feeding. Further studies will be carried out focusing on the elicitation of suspension cell cultures and on the analysis of the effect of elicitation treatment on enhanced production of these secondary constituents.

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