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RESEARCH ARTICLE

BIOTRANSFORMATION STUDIES ON *ENICOSTEMMA HYSSOPIFOLIUM* (WILLD.) VER.

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ABSTRACT

Diosgenin, a steroidal sapogenin found in most of the *Dioscorea spp* is extensively used in the manufacture of cortisone and allied drugs. Increased transport and labour cost and decline in the diosgenin availability due to over exploitation of D*ioscorea spp*. led to deficit supply in world steroid market. Therefore a protocol for production of Diosgenin by biotransformation of cholesterol feeding exogenously as a precursor to the cell suspension and immobilized cell cultures of *E. hyssopifolium* was developed. Prior to the experiment the viability of the cells from node derived callus was tested using fluorescein diacetate. The viable cells that fluoresce yellow upon excitation were used for further studies. Cell suspension and immobilized cell cultures were treated with 50 and 100 mgl^{-1}cholesterol. Cell growth and diosgenin content was examined starting from 8th day to 16th day after culture at intervals of 2 days. The maximum growth index of 1.96 was recorded in cell suspension culture treated with 50 mgl^{-1} after 16 days culture. The highest accumulation of diosgenin was obtained in immobilized cell cultures treated with 100 mgl^{-1} cholesterol on 12th day after culture. In all the cultures, the highest concentration of diosgenin on 12th day where as on 14th and 16th day there was a marginal decline.

INTRODUCTION

Biotransformation reaction is a technique that converts various substrates into a more useful product using freely suspended, immobilized plant cells or enzymes derived from these living cultures or entrapped enzymes. Various precursors and intermediary compounds in biosynthetic pathways have been added to plant cultures to increase the concentration of specific secondary metabolites. Biotransformation reactions are catalyzed by the presence of enzymes present in intracellular location. It may be single step or multi-step reaction and have been shown to occur in callus, suspension cells and or immobilized cultures (**Curtin, 1983**). Growing interest in practical aspect of plant tissue culture led to the exploitation of cell culture systems for the biosynthesis of a variety of natural products, which include alkaloids anthraquinones, phenolics, volatile oils etc. The range of chemicals in plant suspension and immobilized cultures is regarded as potential economical valuable products (**Zenk *et al.*, 1977; Tal *et al.*, 1982; Fujita *et al.*, 1983; Fowler, 1983; Fujita and Tabata, 1987; Kurz, 1989; Charlwood *et al.*, 1986**). Plant cell culture has a number of advantages over conventional procedures. It could ensure a continuous supply of uniform quality and highly specialized natural compound and useful in the study of genetics, biochemistry and physiology of plants. Steroidal sapogenin, diosgenin is synthesized from cholesterol in several plants (**Benett and Heftmann, 1965; Benett *et al.*, 1967; Varma *et al.*, 1969; Stoh's *et al.*, 1974; Eichenberger, 1982; Mahato *et al.*, 1982**). Here we report a cost effective reproducible protocol for biotransformation of cholesterol to diosgenin by cell suspension and immobilized cell cultures of *E. hyssopifolium*.

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MATERIALS AND METHODS

Nodal culture

Top cuttings with 5-7 leaves emanating from a perennial flowering plant in a natural forest segment of the botanic garden, Gulbarga University, Gulbarga. The cuttings were washed in detergent (5% v/v Teepol) for 3-5 min and then in running tap water for 15 min. Surface sterilization was done by immersion in 0.1% (w/v) mercuric chloride for 3-5 min and followed by 4-5 washes in sterile distilled water. Nodal explants (0.5-1.0 cm) were dissected out washed once in sterile distilled water and blotted over sterile filter paper discs before transfer to nutrient medium. The explants were implanted either vertically or horizontally on the medium. The nutrient medium contained salts and vitamins of MS (**Murashige and Skoog's, 1962**) medium with 3% (w/v) sucrose and varied

concentrations of 6-benzyladenine (BA) (0.5-4.0 mgl^{-1}) and α-naphthaleneacetic acid (NAA) (0.5-4.0 mgl^{-1}). The medium was adjusted to pH 5.8 before adding 0.8 % (w/v) agar (Hi Media laboratories Pvt., Ltd., Mumbai), dissolved and dispensed in culture tubes and then autoclaved at 121^0C for 15 min. All cultures were incubated in a culture room maintained at 25 ± 2^0C, RH 50-60% and 14 hr photoperiod. Cool white fluorescent tubes (Philips India Ltd., Mumbai), provided a photon flux density of 2000 Lux.

Suspension culture

Suspension cultures were initiated by transfer of the most friable sectors of an established callus tissue into an agitated 100 ml liquid MS medium (1962) in 250 ml Erlenmeyer flask. The cultures were incubated at 25 ± 2°C on a rotary shaker at 90 rpm under continuous light (2000 Lux,). Cell suspensions were filtered through appropriate sieves to obtain single cells and few celled aggregates which were used as inoculums for sub–culture. The suspension cultures were maintained by sub culturing at 15 days interval by 1:4 (suspension: fresh medium).

Testing the viability of callus tissue

The cell suspension cultures of *E. hyssopifolium* were grown in MS liquid medium and used for determining the viability using fluorescein diacetate (FDA). FDA stock was prepared by dissolving 0.5 g in 100 ml acetone and stored in deep freezer. 0.1 ml of stock was diluted to 5 ml with MS medium and used within one hour for best results.

Immobilization of callus cells

Sodium alginate 1.5% (w/v) (Sd fine chemical Co. Mumbai) was dissolved in hot distilled water and sterilized at 1.4 kg cm^{-2} and 121^0C for 15 min. (1.359 g in 150 ml of water through sterilized glass pipette 4 mm diameter). The 35 days old suspension culture separated from the medium and suspended in about 1 g of a solution of 3% (w/v) sodium alginate to get a final concentration of 1.5% was extruded into $CaCl_2$. The resulting beads were washed thoroughly for 30 min to remove the traces of $CaCl_2$ before transferring to nutrient medium.

Addition of cholesterol

Cholesterol (Sd fine chemical Co., Mumbai) was dissolved in hot ethanol was added to the MS liquid medium at 50 $mgl^{-1}$and 100 mgl^{-1} before autoclaving.

Harvestation

35 days old non diving callus grown on different treatment were harvested after 8, 10, 12, 14 and 16 days of incubation.

Extraction of diosgenin

Cholesterol treated callus were dried in an oven at 60°C and acid hydrolysed with 2 N HCl for 3 hr at 60°C. The mixture was filtered and washed with dilute ammonia. The samples were dried and extracted with ether (60-80°C) in a soxhlet extractor for 16 hr. The solvent was evaporated to dryness *in vacuo* and taken up in methylene chloride.

Quantification of diosgenin

100 mg of pure diosgenin was dissolved in 100 ml hexane. Volumes corresponding to 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg diosgenin were taken into different test tubes, 0.2 ml of test sample was taken in separate test tubes, 5 ml of perchloric acid and 0.1 ml of antimony pentachloride solution (24% in 70% perchloric acid) were added to each test tube. The mixtures were kept for half an hour for the development of red colour. The absorbency of each was noted at 486 nm against blank. The amount of diosgenin in the sample was calculated using standard graph prepared using diosgenin.

RESULTS

Biotransformation of cholesterol at 50 mgl^{-1} and 100 mgl^{-1} in a cell suspension and immobilized cell cultures of *E. hyssopifolium* to produce diosgenin and the growth responses are summarised in Fig. 1 and 2. The friable callus cultures raised from nodal explants of *E. hyssopifolium* (Fig. 3A) on MS medium supplemented with 2,4-D (2.0 mgl^{-1}) + BA (0.5 mgl^{-1}) were used for the suspension culture and biotransformational studies (Fig. 3B and 3C).

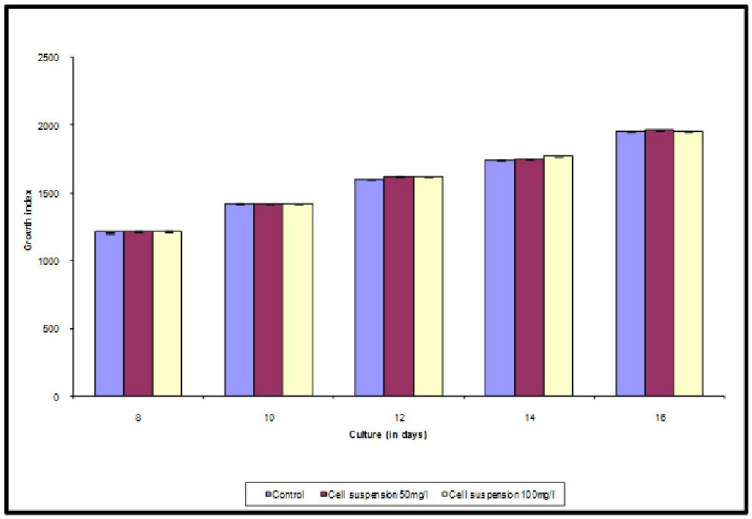

Fig. 1. Growth of cells in the suspension culture of *E. hyssopifolium* fed with cholesterol

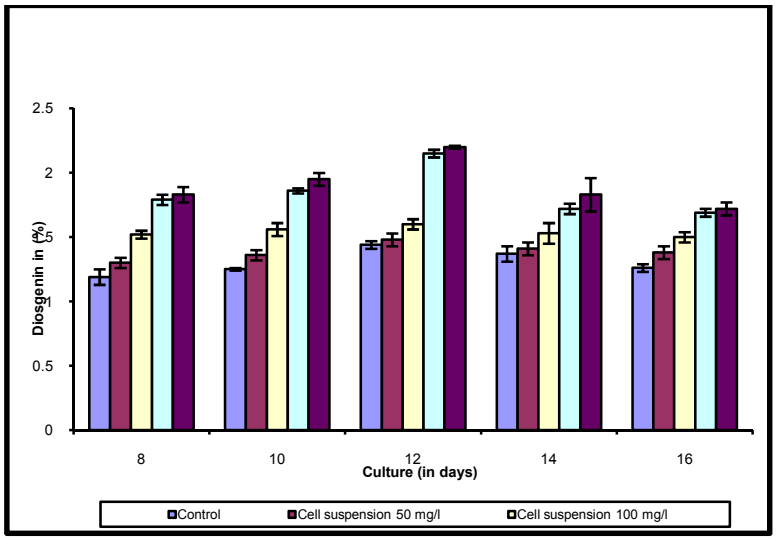

Fig. 2. Biotransformation of cholesterol to diosgenin by cell suspension and immobilized cell cultures of *E. hyssopifolium*

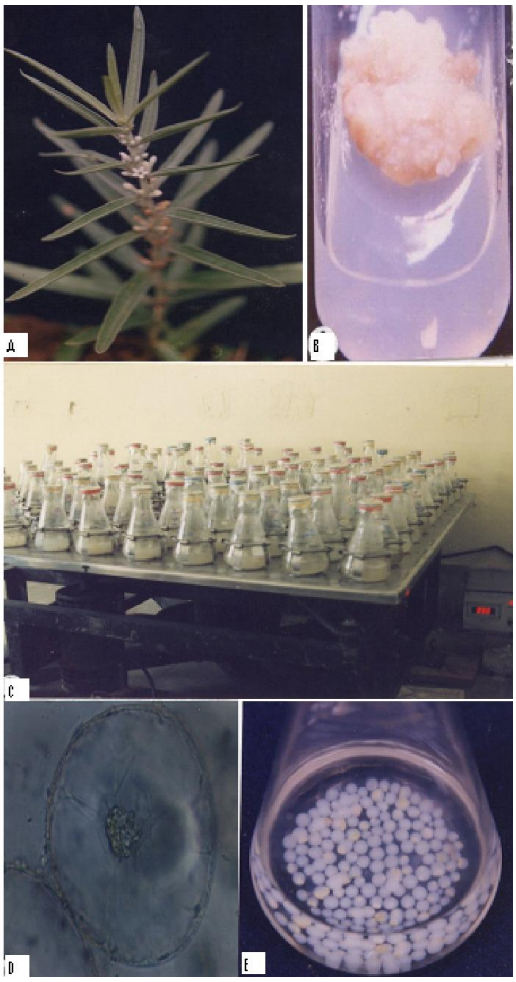

Fig.3.

A. **Habit** - *Enicostemma hyssopifolium* (Willd.) Ver.

B**. Nodal culture** Initiated on MS medium supplemented with 2, 4-D (2.0 mgl^{-1}) + BA (0.5 mgl^{-1})

C. **Suspension culture** Initiated by transferring most friable portion of callus onto 250ml Erlenmeyer flask fed with cholesterol and rotated at 90 rpm.

D. **Viability of cells using fluorescein diacetate** Stereomicroscopic view of viable cell showed yellow colour upon excitation.

E. **Immobilization of callus cells -** Beads of callus cells are coated with a mixture of sodium alginate and $Cacl_2$ in 250 ml Erlenmeyer flask fed with cholesterol.

Viability of cells was assessed using fluorescein diacetate (FDA) as described by Wid-holm 1972. And the cells with yellow florescence were identified as viable cells and used for culturing (Fig. 3D).

Cell growth and diosgenin content were examined in cell suspension and immobilized cell cultures (Fig. 3E) of *E. hyssopifolium* fed with 50 mgl^{-1} and 100 mgl^{-1} cholesterol as a precursor for 8, 10, 12, 14 and 16 days. During the course of 16 days culture no diosgenin was detected in the culture medium suggesting that it was retained within the tissue. Growth index (GI) being 1.96 ± 00 after 16 days culture (Fig. 1). It indicates there is no effect of cholesterol treatment on the cell growth as there is small difference in growth index of both controls and treated. The biotransformation of cholesterol to diosgenin by cell suspension and immobilized culture was maximum on 12^{th} day in all cultures. The highest concentration of diosgenin of 2.20% or 22.05 mg per culture was obtained in immobilized cell cultures treated with 100 mgl^{-1} cholesterol followed by immobilized cell culture treated with 50 mgl^{-1} cholesterol with 2.15% or 21.51 mg per culture. Cell suspension culture treated with 100 mgl^{-1} cholesterol produced 1.60% or 16.05 mg per culture; treated with 50 mgl^{-1} cholesterol 1.48% or 14.85 mg per culture was noticed (Fig. 2). A protocol of diosgenin production in cell suspension and immobilized cell cultures treated with cholesterol as a precursor was shown in flow chart (Fig. 4)

DISCUSSION

Diosgenin is one of the steroidal sapogenin obtained from *Dioscorea spp., Trigonella foenum graceum* and *Costus speciosa.* It is a more useful compound in the steroidal market and useful in the preparation of steroidal drugs such as Betamethasone, Betacortril etc., and oral contraceptives. Earlier, these drugs were prepared using hormones such as oestrodiol from animal source. With the knowledge of diosgenin a chief source from *Dioscorea* was known, these plants are exploited for the extraction of diosgenin and for further synthesis of steroidal drugs. With the advent of cell and tissue culture techniques biotransformation, elicitation,

immobilization and cell culture for the production of useful compounds were used extensively to synthesize compounds of interest.

Biotransformation using cell culture and immobilization has given good results in production of Shikonin and Ginsengoside on a commercial scale. Similar efforts are being made for other such useful compounds of theraupeutical importance. Many compound of medicinal value are being synthesized after knowing their structure and feasibility and demand in market. Diosgenin is one such compound having synthesized and economically feasible. But the demand is ever increasing from the steroidal market. It is around 2100X 10^3 kg/yr. Therefore production of such useful compound to meet the demand using other groups of plants and development of a protocol is imminent. Hence use of *Enicostemma* as a handy and herbaceous plant was undertaken in the present investigation and biotransformed cholesterol to diosgenin was achieved. It has distinct contribution in the production of diosgenin as compared to cultures without precursor. **Khanna *et al.* (1975)** have reported similar positive effect of cholesterol addition on diosgenin production in *Trigonella foenum graecum* suspension cultures. The synthesis of the steroidal sapogenin, diosgenin is not associated with active cell growth and occurred mainly in non dividing cells (**Stoh's *et al.*, 1974; Tal and Goldberg, 1982; Tal *et al.*, 1982**). Prior to the experiment the viability of the cells was assessed using fluorescein diacetate (FDA) as described by **Widholm (1972)**. FDA acts as a substrate for the esterases present in the viable cells, which will cleave FDA into two components of which one is fluorescein, which makes viable cells fluoresce yellow upon excitation, which has advantage over earlier adopted methods of Evans blue **(Graff and O-Agola, 1971).** The viable cells were used for the biotransformational studies.

The presence of nitriloacetic acid, a weak chelating agent in MS medium reduces the precipitation of the medium. The cholesterol was utilized by the cells in the medium as an additional source of precursor for diosgenin synthesis. **Stohs *et al.* (1969)** have added labeled cholesterol to *D. deltoidea* cell suspension and showed that the whole molecule was incorporated into diosgenin. Also, the production of diosgenin was influenced by hormone such as 2,4-D as reported by Marshall and **Staba, (1976).** Therefore, in the present experiment these hormones are added in MS medium in order to increase the production of diosgenin. The maximum accumulation of diosgenin was observed on 12^{th} day in all cultures, which includes control (without precursor), cell suspension (treated with 50 and 100 mgl^{-1}) and immobilized cell (treated with 50 and 100 mgl^{-1}) cultures. The time-course for *in vitro* synthesis of diosgenin varies from plant to plant. The diosgenin concentration was highest on 12^{th} day in *D. delltoidea* **(Ishida, 1988)** and on 14^{th} day in *D. caucasica* **(Deliu *et al.*, 1992)** cell suspension cultures where as in hairy root culture of *Trigonella foenum graecum* the diosgenin production increased relatively slowly during the first 20 days followed by rapid rise for the next 25 days to reach maximum after 45 days **(Merkli *et al.*, 1997).**

It is evident from the results that the accumulation of diosgenin was more in cholesterol treated immobilized cells than in free cells. Similar results have been obtained during capsaicin production using immobilized cells of *Capsicum frutescens* treated with phenylalanine as a precursor **(Johnson, 1990).** Cell culture technology is generally considered to be an efficient and useful to circumvent various problems associated with obtaining natural drugs from field plants. In spite of steady efforts by various scientists, only a few products such as Shikonin and Ginseng are manufactured at large scale. However, the strategies such as immobilization coupled with biotransformation indicate the likely hood of many products to reach commercial levels. The results of present investigation prove to be an efficient protocol to meet the demand of world steroid market.

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