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Effect of culture conditions on synthesis of triterpenoids in suspension cultures of *Lantana camara* L.

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Abstract Present report is aimed to study the batch kinetics of *Lantana camara.* Dynamic changes of parameters, such as pH, conductivity, wet and dry cell concentrations, consumption of major nutrients, carbon source and agitation speeds were investigated to understand the culture characteristics of suspended cells grown on MS + BAP + 2,4-D + NAA in shake flasks. Results indicated that the consumption of phosphate resulted in the onset of stationary phase in cultures. Maltose as carbon source resulted in production of maximum triterpenoid content (31.08 mg/L) while the least was found on glucose (10.69 mg/L). Notably, both did not support accumulation of betulinic acid. Sucrose, although stood second in terms of quantity (21.6 mg/L), supported the production of all the three triterpenoids-oleanolic, ursolic and betulinic acids. Maximum viable cultures were obtained at a rotation speed of 120 rpm. The present finding will form a background for further scale-up related studies.

Keywords Batch kinetics · Cell suspension cultures · *Lantana camara* · Triterpenoids

Introduction

Plant cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals. Particularly, the cell suspension cultures offer a simple system to study growth and production kinetics that can help to evaluate and implement optimal conditions for the production of a number of high value medicinal compounds in good quantities. The Wild Sage or *Lantana camara* L. from the family Verbenaceae has several secondary metabolites, which are therapeutic, and account for its use in traditional medicines all over the world. The plant has been listed as one of the important medicinal plants [1] and used in treatment of various diseases including cancers.

In spite of being a plant of potential medicinal interest, there are very few reports that document the properties of this plant scientifically. Also, no perceptible biotechnological advances have been made in this genus to exploit or enhance its utility. Only a single report by our lab [2] describes the establishment of dedifferentiated biomass from leaves and the cytotoxic activity of its extract on the cancerous HeLa cell lines under in vitro conditions. Apart from this, the existing literature reports focus on the identification of its biochemical constituents and bioactivity from various parts of plants growing in wild [3, 4].

Also, studies on secondary metabolites require an in-depth understanding of biosynthetic pathways which is often difficult to conduct in whole plants because the biosynthetic activities may only be expressed in particular cell types within a specific plant organ or at certain time of season. Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and a condensed biosynthetic cycle. As a result, secondary metabolite production can take place within a short cultivation time (about 2–4 weeks) with an added advantage of tunability [5]. As no reports are available on the dynamics of biosynthetic capacity of *L. camara* cells in suspension cultures, it seemed appropriate to undertake the studies on this aspect. The present study was focused on production of

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three anticancer triterpenoids, betulinic (BA), oleanolic (OA) and ursolic acids (UA).

Materials and methods

Plant material and establishment of suspension cultures

Healthy leaves of *Lantana* were collected from the plants bearing pink–yellow flowers growing at IIT Guwahati campus. Leaves were surface sterilized and cultures were established according to the protocol mentioned earlier [2]. Briefly, they were washed in diluted tween-20 (1:50) for 15 min followed by surface sterilization with 0.1% mercuric chloride for 10 min. After rinsing in sterile distilled water, leaf disc explants were prepared with cork-borer of size 5 mm. The explants were inoculated in Murashige and Skoog (MS) [6] medium supplemented with 6-benzylaminopurine (BAP) (1–5 μM); 3-indole acetic acid (IAA) (5–10 μM); BAP (5 μM) + IAA (10 μM); BAP (10 μM) + IAA (5 μM); BAP (5 μM) + α-naphthalene acetic acid (NAA) (10 μM); BAP (10 μM) + NAA (5 μM); BAP (5 μM) + 2,4-D (1 μM) + NAA (1 μM). The pH of the medium was adjusted to 5.8 before being autoclaved at 121 °C at 15 psi for 15 min. Approximately 20 mL of the medium was dispensed in each test tube. Cultures were maintained in 1,000–1,600 lux light intensity at 25 ± 2 °C.

For establishing suspension cultures, healthy, green, friable and soft callus maintained on the responding semi-solid media was utilized. Erlenmeyer flasks of 250 mL capacity containing 50 mL of liquid medium were inoculated with 0.2 g of fresh cells and incubated under shaking conditions at 25 ± 2 °C in dark.

Carbon source

Three carbon sources viz. glucose, maltose and sucrose were tested at 3% concentration for growth and production profile in suspension culture. The *L. camara* cells were inoculated such that each 250 mL Erlenmeyer flasks containing 50 mL of medium had 0.2 g of the cells. Incubation was done under the culture conditions described above. The flasks were harvested after 16 days to analyze for dry cell weight and triterpenoids content.

Estimation of DCW, phosphate and nitrate

For determination of dry weight, the cells were harvested at an interval of 2 days, washed with distilled water and filtered under vacuum. Thereafter, the cells were dried in oven at 30 ± 2 °C until a constant weight was achieved. The drying temperature was kept low to avert decomposition of thermolabile compounds. The pH and conductivity of the suspension cultures were monitored after every 2 days. Phosphate was estimated by the standard calibration curve made from NaH_2PO_4 [7]. To 0.5 mL of standard or sample solution, 4 mL of reagent (Acetone, 2.5 M H_2SO_4 and 10 mM Ammonium molybdate·$4H_2O$ mixed in the ratio of 2:1:1) was added. The solutions were thoroughly mixed and then 0.4 mL of 1 M citric acid was added. Absorbance was measured at 355 nm. Similarly, for nitrate estimation, standard curve was made from 0.01 N stock solution of KNO_3 preserved in chloroform [8]. After acidification of samples with sulphuric acid, absorbance was recorded at 275 nm in a UV visible spectrophotometer (Cary, Netherlands).

Agitation speed

The effect of different agitation speeds was evaluated in terms of fresh and dry weight generated at the end of each passage and viability of cells. Callus cells weighing approximately 0.2 g were harvested at the end of growth period and re-inoculated in 50 mL of fresh medium of the same composition. The flasks were incubated at 60, 120 and 240 rpm, under darkness, for a period of 3 weeks and their fresh and dry weights were recorded. The viability of cells under each condition was checked with 1% fluorescein diacetate (FDA) solution.

Preparation of plant extracts

The dried cells were soaked in methanol (200 mL) for 48 h, following which the cells were sonicated for 40 min at 30% amplitude (pulser 5 s on/off) and then for next 20 min at the same amplitude (pulser 3 s on/off). After filtration, the methanol extract was centrifuged at 10,000 rpm for 10 min. Supernatant was pooled, filtered and dried in a rotary evaporator at 40 °C. The methanolic extract obtained was further fractionated into an organic (ethyl acetate) and aqueous fractions. The ethyl acetate extract was further dried under reduced pressure in a rotary evaporator at 40 °C (Buchi Rotavapor R-200, Japan). Aqueous fraction was lyophilized and stored at 4 °C until further use.

Estimation of triterpenoids

Quantitative estimation of BA, OA and UA was carried out on Varian Prostar HPLC system (Varian, USA) equipped with a binary pump, UV detector and a 20-μL injection loop. Hypersil BDS RP-C_{18} column (Thermo, USA) of dimensions 250 × 4.6 mm was used with acetonitrile:water (80:20) (v/v) as mobile phase at a flow rate of 1 mL/min. The eluted samples were detected at 209 nm.

Standard compounds of BA, OA and UA were purchased from Sigma (USA) and dissolved in ethanol (Merck) to yield a stock concentration of 5.0 mg/mL. Serial dilution of the standard solutions resulted in concentrations ranging from 0.3 to 2.5 mg/mL. Method linearity was demonstrated by determining a calibration curve for each compound, injecting standards at different concentrations and calculating the regression coefficient (r^2). Slope equation obtained was used to calculate the amount of the triterpenoids in unknown samples.

Statistical analysis

All results reported are an average of two separate analysis for triterpenoid estimation and two consecutive experiments with three replicate flasks in each treatment for kinetics of growth and effect of other variables like pH, conductivity and nutrient consumption. Results are represented as mean ± SD. Specific growth rate (μ) was calculated by:

$$\mu = \mathbf{ln}(MT_2 - MT_1)/T_2 - T_1;\ T_2 > T_1$$

where, MT_2 and MT_1 are biomasses at the different time points (T_1 and T_2), respectively.

Results and discussion

Establishment of cell lines

Almost all the media combinations tested for leaf-disc dedifferentiation resulted in browning and death of explants soon after inoculation. Only MS medium supplemented with BAP (5 μM), 2,4-D (1 μM) and NAA (1 μM) was found appropriate for callus differentiation of the explants. On this medium, the leaf discs resulted in bright-green, hard, compact calli from the cut margins after a week of culture. When this compact calli were taken further into the subculture cycles, browning of cultures was observed due to high phenolic content. Regular subculturing at 4-week intervals, for about 26 weeks, reduced browning, improved the nature of callus and rate of callus proliferation.

Suspension cultures were initiated once the stable cell lines were obtained on semi-solid medium. The same growth regulator combination (BAP + 2,4-D + NAA), as used for callus culture worked for cell suspension cultures. However, in suspension cultures the growth period was reduced to 3 weeks as compared to 4 weeks required in the case of callus growth on semi-solid medium. Reduced growth period might be due to facilitated nutrient transport in liquid medium [9]. Again, the dark incubation was found more suitable for suspension cultures.

Kinetics of cell growth and nutrient uptake

It was observed that the cultures remained in the lag phase till the second day. Biomass increased till the tenth day following which the stationary phase started (Fig. 1). With the formula mentioned in "Materials and methods" section, the specific growth rate (μ) of the suspended cells was found to be 0.1072/day. The pH of the medium underwent variation during different stages of culture. It was observed

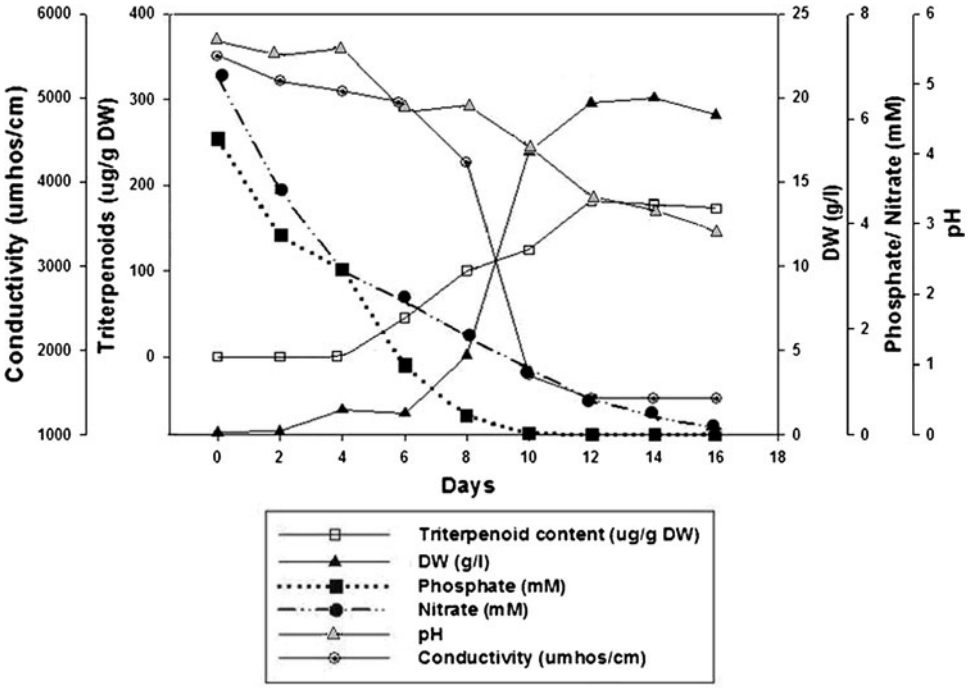

Fig. 1 Dynamics of various variables in cell suspension cultures of *L. camara* cells

Table 1 Effect of different carbon sources on growth of cells and production profile of triterpenoids in cell suspension cultures

S. no.	Carbon source	DW (g/L)	Total triterpenoid content (mg/L)
1	Glucose	92.8 ± 1.8	10.69 ± 0.2
2	Maltose	105.8 ± 2.1	31.08 ± 0.5
3	Sucrose	117.8 ± 2.0	21.6 ± 0.6

that after showing a slight decrease, the pH dropped sharply between 4–6 days, which dropped further after 10 days (Fig. 1). This may be attributed to preferential uptake of NH_4^+ ions which resulted in decreased pH due to liberation of H^+ ions; pH tends to increase if NO_3^- is utilized faster than NH_4^+ [10]. Also, concomitant synthesis of acid triterpenes in the medium may be the reason as their synthesis was found to be mainly growth-associated. Conductivity, as expected, showed an inverse relationship with growth. Among the major inorganic nutrients, it was invariably observed that phosphate was almost completely consumed by the tenth day of culture. Its utilization was very fast in the initial days than in the later stages of growth. Uptake of nitrate was at slower rate in comparison to phosphate. It was present in the culture medium till the last day of cultivation (16th day) (Fig. 1). Hence, it may be concluded that complete utilization of phosphate from culture medium resulted in the onset of stationary phase and it was a major limiting nutrient for growth. Similar kind of kinetic profiles, where phosphate is assimilated faster than the nitrate were observed and reported by many workers in different plant species [11, 12].

Effect of carbon sources on synthesis of triterpenoids

The presence of three acid triterpenoids was established in our preliminary studies with callus cultures (data not shown). Extension of the same protocol for extraction and HPLC analysis, where the cells were harvested at every alternate day from liquid suspension cultures, revealed that the synthesis of these triterpenoids is concomitant with the log phase of cultures. On comparison with the standard compounds, it was found that BA, OA and UA eluted at 10.8, 12.6 and 13.2 min, respectively. With the onset of stationary phase, the amount of these acids showed a decline in the medium, while in the lag phase they were not detectable. Similar growth-coupled synthesis of triterpenoids has been observed previously in case of other triterpenoid, azadirachtin [13].

The nature of carbon source in the medium bears profound effect on type and quantity of metabolite produced. Maltose gave the maximum yield (31.08 mg/L) of triterpenoids, followed by sucrose (21.6 mg/L) and glucose (10.69 mg/L) (Table 1). However, it is noteworthy that in maltose and glucose, BA was totally absent and the values represented, here, are OA and UA. Sucrose, though second best in terms of amount, favoured the production of all the three triterpenoids (Fig. 2). Variation in growth and chemical profile in in vitro cell cultures with manipulation of media components has been previously reported in other plant species [14]. Also, the positive influence of maltose on quantity of metabolites has also been reported by others. For example, Kinnersley [15] reported twice as high production of anthocyanin in cells grown on maltose as the ones grown on sucrose. Choi et al. [16] reported that intermittent feeding of maltose considerably enhanced the paclitaxel production in comparison to sucrose. However, as far as the growth of *L. camara* cultures is concerned, maximum biomass was obtained in sucrose (1,018.2 g/L) which was best in terms of callus proliferation and multiplication, followed by maltose (616 g/L), whereas, least biomass was obtained in glucose (253 g/L). Similar kind of effect on biomass production has been observed in cell suspension cultures of *Psoralea corylifolia* [17].

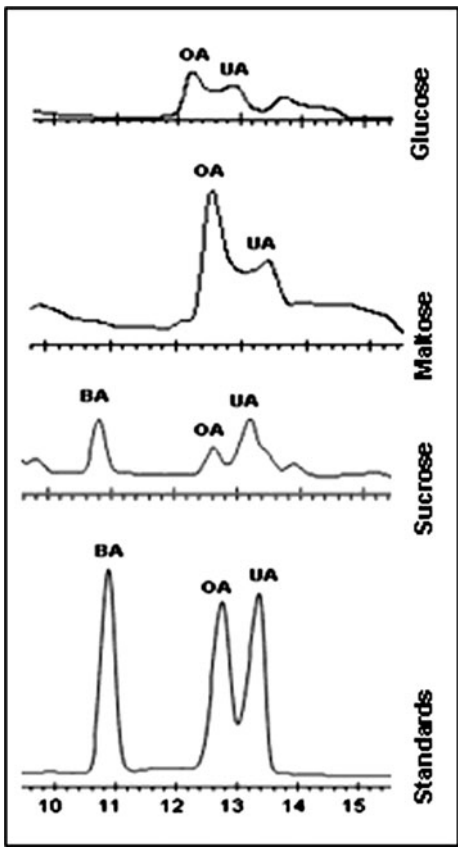

Fig. 2 HPLC chromatograms representing the presence/absence of three terpenoids in cultures grown on sucrose, glucose and maltose. Standard sample (Sigma) was analyzed after mixing all the three acid terpenoids—betulinic acid (*BA*), oleanolic acid (*OA*) and ursolic acid (*UA*)

In accordance with their results, our studies also demonstrate the effectiveness of sucrose for biomass production. The results are also in agreement with a recent finding which says that sucrose being a balanced source of carbon supply favours highest biomass accumulation in *Dendrobium huoshanense* cell suspensions cultures [18].

Effect of agitation speed on cell survival and viability

Speed of agitation directly affects the growth and viability of cells in culture due to aeration and agitation. In the present study, maximum fresh weight (1,018.2 g/L) and maximum viability (Fig. 3b) was observed at 120 rpm. In the remaining two agitation speeds, the biomass was more or less the same but much less than at 120 rpm. The viability profile showed that at 60 rpm cells died due to aggregation and clumping; only the cells that formed the outermost layer of the aggregate were alive (Fig. 3a). At 240 rpm, the cells died due to rupturing (Fig. 3c). FDA is a non-fluorescing, non-polar dye that freely permeates through the plasma membrane. In the living cells, it is cleaved by esterase activity releasing the polar fluorescent portion, fluorescein, which is unable to pass through the plasma membrane of living cells while in dead and broken cells it is lost. Hence, only the live, intact cells take up the stain and fluoresce green. The perceived sensitivity of plant cells to hydrodynamic stress associated with aeration and agitation can be attributed to the physical characteristics of the suspended cells, viz. their size, the presence of thick cellulose-based cell wall and existence of large vacuoles [19].

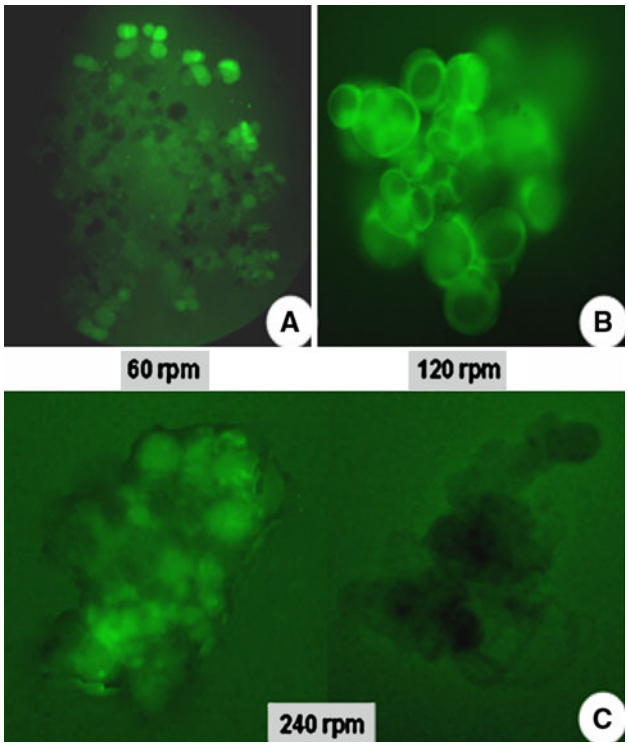

Fig. 3 Effect of agitation speed on growth and viability of cells; 3-week-old cells stained with 1% fluorescein diacetate solution. **a** Cellular clump at 60 rpm showing unstained dead cells in the centre of cell aggregate and live cells fluorescing green at the periphery. **b** Cultures maintained at 120 rpm showing small cell aggregates of live and healthy stained cells. **c** Cultures maintained at 240 rpm showing dead clumps (*dark bodies*) and sheared cells

Conclusions

The present study broadly elucidates all the parameters and conditions necessary for establishment of suspension cultures of *L. camara*. The synthesis of triterpenoids was found to be growth associated. Phosphate was found to be the growth limiting nutrient as its complete consumption lead to the onset of stationary phase in the cultures. Among the carbon sources, sucrose supported maximum biomass production and production of all the three acid triterpenoids. Studies form a background for future studies related to scale-up of cultures in bioreactors.

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