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Original Article

Investigation on Production of Camptothecin and its Analogues in tissue culture of *Ervatamia heyneana*

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ABSTRACT

Tissue cultures of the leaves and stems of the indigenous tree *Ervatamia heyneana* (Wall) T. Cooke were established with the aim to investigate the production of camptothecin (CPT) and its analogues such as 9-methoxy camptothecin and 10-hydroxy camptothecin. Unorganized callus tissue was developed from stems on Murashige and Skoog's (MS) medium, whilst Gamborg's (B_5) medium was used to develop unorganized callus tissue from leaves containing picloram 0.2 mg/L and benzyl adenine 0.2 mg/L. Accordingly, cell suspension cultures were established on the respective media composition from callus after 4 passages. Growth indices of cell suspension cultures were calculated and maintained by sub-culturing every 15 days. After maintaining for 2 passages, aliquots of sample were withdrawn from cell suspension cultures every 3 days up to the 15th day and filtered to separate medium and cells. Methyl jasmonate, arachidonic acid, cadmium chloride and silver nitrate used as elicitors were separately added to cell suspension cultures in two different concentrations (100 µm/L and 500 µm/L) on day 7, and were filtered after completion of the15 day incubation period. Callus cultures (8, 12 and 16 weeks old), cells and the medium from cell suspension cultures were extracted and analyzed for CPT and its analogues. But none of the extracts showed the presence of CPT or its analogues.

Key words: Tissue cultures, *Ervatamia heyneana*, Camptothecin, Elicitors, Growth indices.

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INTRODUCTION

Due to their complex structure with several chiral centers some important anticancer agents are still extracted from plants and not synthesized chemically on a commercial scale. Sustainable production of bioactive compounds of interest may be achieved by plant *in vitro* cultures. Camptothecin (CPT) is a modified monoterpene indole, anticancer and antiviral alkaloid produced by *Camptotheca acuminata* (Nyssaceae),[1] *Nothapodytes foetida* (Icacinaceae),[2,3] some species of the genus *Ophiorrhiza* (Rubiaceae),[4,5] *Ervatamia heyneana* (Apocynaceae),[6] *Merrilliodendron megacarpum*,[7] *Pyrenacantha klaineana* (Icacinaceae),[8] *Mostuea brunonis* (Gelsemiaceae)[9] and *Chonemorpha grandiflora* (Apocynaceae).[10] Production from natural resources, however, has been problematic because of the uncertainty of cultivation due to weather changes, pests and disease. Production of CPT and its analogues through tissue culture is therefore one of the alternative methods for obtaining these high cost compounds. A number of attempts have been made for sustainable production of CPT and its analogues from *in vitro* cultures of *C. acuminate*,[11,12] *N. foetida*,[13,14] *Ophiorrhiza pumila*,[15] *O. mungos*,[16,17] *C. grandiflora* etc.[10]

In the present study, the possible production of CPT and its analogues such as 9-methoxy camptothecin (9MCPT) and 10-hydroxycamptothecin (HCPT) by tissue cultures of *E. heyneana* (Wall) T. Cooke has been investigated. *E. heyneana* is a flowering shrub, widely grown along the coastal regions of Western India.[18] Alkaloids and triterpenoids are the major classes of compounds isolated from this plant. The pyrroloquinoline alkaloids CPT and 9MCPT are two important alkaloids isolated from wood and stem bark of this plant and exhibit cytotoxic activity.[6,19] To the best of our knowledge there are no reports on the production of CPT and its analogues 9MCPT and HCPT from *in vitro* cultures of *E. heyneana*.

MATERIALS AND METHODS

Plant material

Young leaves and stems were collected from plants grown at the University College of Pharmaceutical Sciences' garden, Kakatiya University campus, Warangal, AP, India. They were washed with tap water followed by detergent solution and again rinsed with running tap water. The explants were then washed with 10% v/v Tween 20 for 5 min followed by thorough rinsing with distilled water. They were again washed with alcohol (70% v/v) for 1 min in aseptic chamber, the alcohol decanted, and explants washed with sterile distilled water 2-3 times. Finally, they were treated with mercuric chloride solution (0.1% w/v) for 5-6 min followed by four to five times washing with sterile distilled water.

Callus initiation

The surface sterilized explants were cut into small pieces (1 cm^2) with the help of sterile scalpel and incisions were made on explants. Then they were transferred onto Murashige and Skoog's (MS) and Gamborg's (B_5) agar media supplemented with different combinations of picloram (0.05 mg/l, 0.1 mg/L, 0.2 mg/L) and benzyl adenine (0.05 mg/L; 0.1 mg/L, 0.2 mg/L) and sucrose 3% w/v. The medium was gelled with 1.1% (w/v) agar-agar. The pH of the medium was adjusted to 5.7-5.8 with 0.1N NaOH before autoclaving at 15 lb, 121°C for 20 min. The cultures were incubated in dark at a temperature of 25-28°C.

Growth index

To evaluate the effect of media composition on callus induction and alkaloid production, callus induced on each of the medium was maintained by sub-culturing on the same medium and conditions every 30 days. The growth index was determined at an interval of one week up to 6 weeks.

Extraction

Callus cultures of 8, 12 and 16 week old were taken for extraction. An accurately weighed quantity (0.5 g) of callus was dried in an oven at 60°C. After complete drying the material was powdered and extracted with 3 volumes of methanol for overnight. This process was repeated 3x for complete extraction. The combined methanolic extract was evaporated, dissolved in 5 mL of water and extracted 3× each with 5 mL of chloroform. The combined chloroform extract was evaporated to dryness. Then the residue was dissolved in 1.5 mL of HPLC grade methanol and the resulting solution was subjected to centrifugation at 10,000 rpm at 20°C for 20 min. Then 1 mL of supernatant was transferred into eppendorff tube and stored in a refrigerator until analyzed. Extraction was performed by taking the cultures in triplicate.

Establishment of cell suspension cultures

About 5% w/v of callus after 4 passages was used as an inoculum for initiation of cell suspension cultures of *E. heyneana*, in the respective media. The cultures were maintained at 120 rpm in refrigerated shaker incubator at 25 ± 2°C in dark.

Growth and production kinetics

From the cell suspension cultures, aliquots were withdrawn on 0, 3rd, 6th, 9th, 12th, 15th and 18th day, filtered and weighed for determination of growth kinetics of the culture. This procedure was done using triplicate flasks and growth indices were calculated. The cultures were sub-cultured onto the same respective medium at an interval of 15 days.

Cells and medium were extracted with 3 volumes of dichloromethane for overnight. This process was repeated 3x for complete extraction. The combined dichloromethane extract was evaporated to dryness and residue was dissolved in 1.5 mL HPLC grade methanol. The resulting solution was subjected to centrifugation as mentioned above for callus. Then, 1 mL of the supernatant was separated into eppendorff tube and stored in a refrigerator. The procedure was performed in triplicate.

Addition of elicitors to cell suspension cultures

Methyl jasmonate and arachidonic acid were dissolved in ethanol. Cadmium chloride and silver nitrate were dissolved in sterile double distilled water. All the elicitors were separately added to cell suspension cultures after 2 passages to final concentrations of 100 µM/L and 500 µM/L, on day 7 of the incubation period while running the control. The flasks were incubated in a refrigerated shaker incubator at 25 ± 2°C and 120 rpm for 15 days. Cells and medium of cell suspension cultures were harvested 15 days after incubation and were extracted for CPT and its analogues as indicated for cell suspension cultures.

Analysis

Thin layer chromatography (TLC)

The concentrated extracts of callus, cells and media from cell suspension cultures before and after addition of elicitors were co-chromatographed with reference samples of CPT, 9MCPT and HCPT on pre-coated silica gel G plates using chloroform-methanol (24:1) as a solvent system and viewed under UV light.[13]

High pressure liquid chromatography (HPLC)

HPLC analysis was carried out using Shimadzu, LC-10AT model, ODS, Nucleosil 100, RP, C_{18} column of 25 cm length, 0.4 cm internal diameter and 5 µm particle size and analyzed at 254 nm by photodiode array detector (model SPD-M10Avp). Samples were eluted with MeOH: H_2O (60:40) at a flow rate of 1 mL/min by injecting 20 µL of sample. The presence of CPT, 9MCPT and HCPT in samples was checked by comparing the retention times.[13,20]

RESULTS AND DISCUSSION

In the present study callus and cell suspensions cultures of *E. heyneana* have been initiated and established. Leaf and stem segments were taken as explants for culture establishment. Callusing response was obtained on both MS and B_5 media. For initial callus induction, it was observed that a combination of auxin and cytokinin was necessary. Once induced, the calli exhibited growth response in media containing either type of hormones. Good callusing response of stem explants on MS agar and of leaf on B_5 agar medium supplemented with phytohormones picloram 0.2 mg/L and benzyl adenine 0.2 mg/L and sucrose 3% w/v was obtained. Callus initiation was observed on the 5th day. Callus growth was profuse and the entire surface of the explants was covered with callus. White to light creamy friable callus was observed. These media were used for further studies as all other combinations of phytohormones concentrations produced poor to negligible amount of callus. Growth index of callus showed that there was an increase in weight of callus until the 4th week and thereafter there was a decline. Hence, callus cultures were maintained by sub-culturing at an interval of 4 weeks onto fresh medium of same composition. Callus of 8, 12 and 16 week old was extracted and analyzed for CPT and its analogues.

Suspension cultures were initiated using 4 week old callus in same media composition. They were maintained by sub-culturing at 15 days interval, as there was an increase in weight of suspension culture from 6th day, which reached maximum on day 15. Abiotic elicitors were added to cell suspension cultures after two passages on 7th day of incubation period. These were extracted in similar way to those of the suspension cultures.

TLC analysis of extracts of 8, 12, 16 week old callus, cells, medium of cell suspension cultures and elicitor treated cell suspension cultures revealed that neither CPT nor its analogues were produced in these *in vitro* cultures. The inability of these *in vitro* cultures to produce CPT and its analogues was also confirmed by HPLC analysis. These findings demonstrated a remarkable note that the callus tissue or cultured cells or elicitor treated cells show no indication for the presence of alkaloids after several sub-culturings of callus or final establishment of well growing cell suspensions.

It is widely recognized that cultured plant cells represent a potential source of phytopharmaceuticals but very few cell suspension cultures synthesize secondary metabolites. There are a number of factors that affect the biosynthetic capacity of *in vitro* cultures like tissue origin, genetic character, culture conditions etc. Plant cells are genetically totipotent. By using the right culture medium and appropriate phytohormones, it should be possible to establish *in vitro* cultures of almost every plant species. Starting from callus tissue, cell suspension cultures can be established that can even be grown in large bioreactors. Therefore, proper environmental conditions should be given so that any cell may be induced to produce any substance according to the characteristic of parental plants. Although, in some cases it has been found that low yielding plants produce high amounts of products and *vice versa*, in general, plant parts that contain highest concentration of the desired product(s) should be chosen.

The two major problems concerned with callus and cell culture are poor expression of products and instability of cell lines. Cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant and these quantitative and qualitative features may change with time. The poor product expression is often attributed to a lack of differentiation in cultures. On the other hand, there are cases of cultures that overproduce metabolites compared with the whole plant. In some instances they may also produce secondary metabolites that are not present in the intact plants. For example, anthraquinones of shikimate pathway are widely distributed in Rubiaceae

plants and a number of cell suspension cultures have been reported to produce these compounds. Phytochemical investigation of *Ophirorrhiza pumila* wild plant did not show the presence of anthraquinones. But, the *in vitro* grown callus and cell suspension cultures established from cells and tissues of the same plant produced anthraquinones but none of the camptothecin-related alkaloids.[21] Similarly, *Cinchona* plants do not contain anthraquinones, whereas *C. ledgeriana* and *C. pubescens* produced these metabolites when their cells were cultured *in vitro* as callus or cell suspension cultures.[22,23] These findings reveal that there is a remarkable difference in constituents between the wild plants and the callus tissue or cultured cells. It has become apparent that the choice of original plant material having high yields of the desired phytochemical may be important in establishing high-yielding cultures.

It has been well documented that the chemical composition of nutrient media influences the growth of biomass and synthesis of secondary products. A balance should be maintained between the production of biomass and secondary products. However, excess increase in biomass reduces the yield of desired products. The major chemicals that affect biomass are carbohydrates and their different sources, nitrogen, potassium, phosphorus, trace elements, vitamins, etc. Among the number of components of medium, phytohormones such as auxins and cytokinins show remarkable effects on growth and biosynthetic ability of plants. There are reports on the effect of medium composition on secondary metabolite production in several plant species like shikonins in *L. erythrorhizon,*[24] plumbagin accumulation in *Plumbago rosea* and *Drosophyllum lusitanicum,*[25,26] pigment betalain in *Beta vulgaris*[27] and indole alkaloids in *Catharanthus rosues.*[28,29]

Physiological factors such as light, temperature, pH, etc. do also affect the product yield. The production of secondary metabolites can be stimulated and enhanced by optimizing the illumination conditions, such as the light quality and intensity and combination of light sources, photoperiod and dark conditions. It has been reported that CPT production is stimulated by light illumination, but cell growth was suppressed. Among the light condition tested, dark condition gave the highest value on cell dry weight and growth index, whereas highest CPT yield was obtained in green light. CPT was not produced from callus in dark condition. Impulse of a particular wavelength of light triggers morphological or biochemical differentiation process.[30] The photoperiod had no effect on cell growth and CPT yield. This may be because photoperiod was favorable to CPT biosynthesis.[31]

Cell and organ cultures have been widely employed to study the formation of secondary metabolites in large number of plants. Unfortunately biosynthesis of phytochemicals in plants and in tissue cultures is often dependent on the presence of differentiated tissues or the development of specialized cells. This situation often occurs when the metabolite of interest is produced only in specialized plant tissues or glands in the parent plant. For example, many sesquiterpene lactones of the Asteraceae produced in trichomes, saponins in roots of ginseng, hypericins and hyperforins in foliar glands of *Hypericum perforatum* (St. John's wort) have not demonstrated the ability to accumulate phytochemicals in undifferentiated cells.[32] A close correlation exists between the expression of secondary metabolism and morphological and cytological differentiation, but it is not yet clear to what extent secondary metabolism depends on the development of specific structures, it is unknown whether these two processes are genetically and/or physiologically linked.[33]

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses.[34] Hence in an attempt to induce secondary metabolite synthesis, abiotic elicitors methyl jasmonate, arachidonic acid, silver nitrate and cadmium chloride in two different concentrations were added to cell suspension cultures on 7th day of incubation period of cell suspension cultures. But these could not induce secondary metabolites synthesis. Inability to induce the production of secondary metabolites might be due to factors like age of culture used for elicitation, concentration of elicitors, duration of elicitor exposure, elicitor specificity, nutrient composition of medium and environmental conditions.

CONCLUSION

Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants. However, only a few cultures produce these compounds in commercially useful amounts. On the other hand many reports have described that yields of desired products are very low or sometimes not detectable in undifferentiated cells such as callus tissues or suspension cultured cells. The low productivities are associated with our poor understanding of the biochemistry of these systems. Recent advances in molecular biology, enzymology, physiology, and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products. The expression of biosynthetic pathways can be influenced by optimization of the cultural conditions such as optimizing production medium by selecting media components and phytohormones, pH, environmental conditions like temperature, illumination conditions, such as the light quality and intensity and combination of light source, supply of precursors, and the application of elicitors. Development of a certain level of differentiation is also considered to be important in the successful production of phytochemicals by cell suspension cultures. Therefore efforts have to be made to stimulate or restore biosynthetic activities of cultured cells using various cultural methods.

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