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ENHANCEMENT OF TYLOPHORIN *IN VITRO* TISSUE CULTURE OF *TYLOPHORA INDICA* (ASCLEPIADACEAE)

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This research paper highlights the enhancement of tylophorin ,a phenanthroindolizidine alkaloid *in vitro* tissue culture of *Tylophora indica*, which is an antiasthmatic plant. In the present study, the effect of BAP, Phenylalanine and Zenk medium were examined on the production of tylophorin in tissue culture of *Tylophora indica.* BAP, and Phenylalanine were incorporated with MS medium in different concentration separately ,while Zenk media was used as a production media. MS medium supplemented with 2ppm of NAA and 0.2 ppm of BAP was used to initiate the callus from leaf explant. When the different concentrations of phenylalanine (25,50,75 and 100 mg /100ml) were incorporated in MS medium separately ,the tylophorin content increased. Six weeks old callus ,fed by 75 mg /100ml of phenylalanine was showing the maximum enhanced content of tylophorin(0.342%), that is almost equal to tylophorin content(0.391%) of leaves. The effect of BAP on production of tylophorin *in vitro* was also enthusiastic (control-0.096% ; six weeks old BAP fed -0.273%) whereas Zenk production media was showing the best results (control- 0.096% ;0.696% in six weeks old culture) which is almost six fold higher than control, and around two fold increase than that of leaves.

Keywords : BAP; Callus; Phenylalanine; *Tylophora indica;* Zenk media.

Introduction

Plant cells cultured *in vitro* have been considered as a potential source of specific secondary metabolites (medicinally important compounds). Cell cultures may contribute in at least four major ways to the production of natural products. These are as: (i) A new route of synthesis to established products e.g. codeine, quinine, pyrethroids, (ii) A route of synthesis to a novel product from plants difficult to grow or establish *e.g. Papaver bracteatum,* (iii)A source of novel chemicals in their own right *e.g.* rutacultin from culture of *Ruta,* (iv)As biotransformation systems either on their own or as part of a larger chemical process e.g. digoxin synthesis. This aim can be achieved by selection of specific cells producing high amount of desired compounds and the development of a suitable medium. The secondary metabolites are known to play a major role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals[1]. Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years. The scheme of production of some important plant pharmaceuticals produced in cell cultures[2] . Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformation of natural compounds has been demonstrated .Using this methodology a wide range of chemical compounds have been synthesized[3]. L-3,4-dihydroxyphenylalanine, is an important intermediate of secondary metabolism in higher plants and is known as a precursor of alkaloids,which has been used for production of alkaloid in *Vinca, Mucuna, Baptisia* and *Lupinus*[4]. Productivity of berberine was increased in cell cultures by optimizing the nutrients in the growth medium and the levels of phytohormones. By selecting high yielding cell lines, Mitsui group produced berberine on a large scale.

Biotransformation is a process through which functional groups of organic compounds are modified by living cells. Biotransformation done by plant cell culture system can be desirable when a given reaction is unique to a plant cell and the product of reaction has a high market value. The useful natural products are synthesized through secondary metabolism, hence they are also known as

secondary metabolites. During metabolism in growing cells, the secondary metabolites are either deposited in vacuoles or excreted from gland cells. Large scale yield of secondary metabolites from cultured plant cells can be increased simply by changing the physiological and biochemical conditions from growth medium. Several factors like growth condition ,organogenetic potential and precursor addition are known to influence product biosynthesis[5]. The leaves and roots of *Tylophora indica* have emetic, cathartic, laxative, expectorant, diaphoretic and purgative properties. It has also been used for the treatment of allergies, cold, dysentery, hay fever and arthritis. It has reputation as an alterative and as a blood purifier, often used in rheumatism and syphilitic rheumatism. Root or leaf powder is used in diarrhea, dysentery and intermittent fever. Dried leaves are emetic diaphoretic and expectorant. It is regarded as one of the best indigenous substitute for ipecacuanha. It is traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, inflammation, bronchitis, allergies, rheumatism and dermatitis. It also seems to be a good remedy in traditional medicine as anti-psoriasis, seborrhea, anaphylactic and leucopenia. Tylophorin, an anti asthmatic and anti cancerous phenanthro indolizidine alkaloid[6] is the main constituent of *Tylophora indica,* has been studied in tissue culture by many scientists[7,8]. The callus initiation of *T.indica* has been documented by using different medium proportion[7,8]. Growth inhibition is often associated with cyto differentiation and the induction of enzymes for secondary metabolism, so dual culture system is preferred. Dual culture system involves biomass production in a medium optimum for cell proliferation followed by transfer of healthy cells to a different medium which is favorable for product yield. This strategy was used by Zenk *et al.*[9] for the production of indole alkaloids by *Catharanthus roseus* cells. In the present study, this strategy was used to enhance the production of tylophorin in *Tylophora indica.* Cytokinin concentration in the media also supports the enhancement of secondary metabolite. Khanna *et al.*[10] have reported the enhancement of secondary metabolite by feeding the callus with phenylalanine in various medicinal plant sps. So with all this background we have selected BAP (Benzylamino purine) , Phenylalanine and Zenk production media to enhance the production of tylophorin in callus of *Tylophora indica.*

Material and Methods

The plant material collected from the Kelkar farmhouse, Mulund, was used for the initiation of callus. The young leaves of the plant were surface sterilized with 0.1% of mercuric chloride and washed with sterile double distilled water. The surface sterilized leaves were cut in to pieces (1 cm) and aseptically inoculated in to the sterilized MS (Murasinge and skoog's) basal media supplemented with 3 % of sucrose, 2ppm of NAA and 0.2 ppm of BAP. The media was solidified with 0.7 % of agar. It was adjusted to 5.8 pH before autoclaving (121^{0}C for 15 min) .Various other hormones like 2,4D,Kinetin ,IAA ,IBA in different combination were also used but 2ppm of NAA and 0.2 ppm of BAP gave the best results. The cultures were incubated at 25^{0}C at 8 hours of dark and16 hour of photo period (1000 Lux). After twenty days of incubation proliferation of leaves skin can be marked and finally bursting of the epidermis can be seen .The callus initiation started from midrib portion of the leaves. Sub culturing in to fresh media was carried out at the time interval of 4 weeks (Fig.1 A-D). The developed callus was then transferred to various media *i.e* MS+ Phenylalanine (25,50,75,100 mg/100ml), MS +BAP (1,1.5,2,2.5 ppm) and Zenk production media without growth hormone separately. Leaves and all the callus samples were dried, powdered, weighed and subjected for extraction using methanol. Thin Layer Chromatography was carried out using the solvent system of Toluene: Ethyl acetate: Diethyl amine (14 : 2 : 2) corresponding with that of standard compound of tylophorin (Alexis Co. New Delhi). Developed plates were sprayed with Dragendorff's reagent. A characteristic brick red color corresponding to standard compound showed the presence of tylophorin alkaloid.

HPTLC Analysis-HPTLC analysis was carried out in Anchrom Test Lab Pvt. Ltd using silica gel plates (60F254 Manufacturer E. MERCK KGaA),Sample application was carried out on CAMAG Linomat 5 Instrument (CAMAG Linomat 5 "Linomat5_080222" S/N 080222).Inert gas was used as spray gas. Sample solvent type was methanol. Dosage speed was 150nl/s and syringe size was 100µl and the analysis wave length was 430 nm. Toluene: Ethyl acetate: Diethyl amine (14:2:2) was used as mobile phase (Fig.5A-B).

Results and Discusion

Alkaloids are cyclic nitrogen containing secondary metabolites[11], where the nitrogen atom is derived directly from an amino acid[12]. Alkaloids are biosynthesized by many unique metabolic pathways and displayed a broad spectrum of pharmacological activities[13].Secondary metabolites are produced in small amount where enzymatic and genetic evidence exist for these pathway[14]. The 60% of drugs from plant origin are alkaloids[15]. *Tylophora indica* is a climbing perennial plant indigenous to India, where it grows wild in the southern and eastern regions and has a

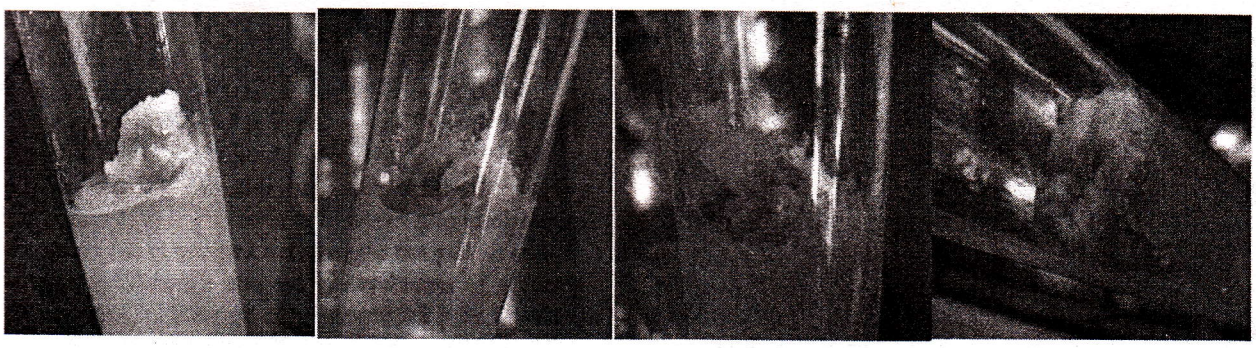


Fig.1. Developmental stages of callus (A-D) initiated from leaf explants in *Tylophora indica.*

long-standing reputation as a remedy for asthma (hence the name, *T. asthmatica*). It has been used for the treatment of various respiratory problems besides asthma, including allergies, bronchitis and colds, as well as dysentery and oseteoarthritis pain.

An important limiting factor in the application of tissue culture for synthesis of metabolites at the industrial level is the composition of the medium (production medium)that promotes the production of desired compound, that generally does not effect the growth of the callus therefore, a two step approach is desirable where one medium is solely for biomass growth and second for accumulation of secondary metabolite minimize the phosphate content in the medium can stimulate production of secondary metabolite production[9]. Many times treatment with precursor and growth regulator like cytokinin effects the production of secondary metabolite production[16]. In the present study, we described the enhancement of tylophorin alkaloid in callus initiated by leaf explant on MS medium supplemented with 2ppm of NAA and 0.2 ppm of BAP. When the different concentrations of phenylalanine (25,50,75 and 100 mg / 100ml) were incorporated in MS medium separately, the callus that fed by 75 mg /100ml was showing the enhanced content of tylophorin (0.342%), that is almost equal to tylophorin content(0.391%) of leaves. The effect of BAP on production of tylophorin *in vitro* increased the content up to some extend (control-0.096%; BAP fed -0.273%) whereas Zenk production media was showing the best results (control- 0.096%; 0.696% in six weeks old culture in Zenk media), which is almost six fold higher than control, and around two fold increase than leaves (Table 1-3; Fig.1-3).

Table 1 & Fig.2. Showing the tylophorin content increased by feeding the callus by different concentration of phenylalanine of *Tylophora indica.* Mean value ± s.e. of three replicate.

S. No.	Media used	Tylophorin content (%)
1	Phe.al. fed MS+25mg/100ml	0.136±0.054
2	Phe. fed MS+ 50mg/100ml	0.241±0.058
3	Phe. fed MS+75 mg/100ml	0.342±0.023
4	Phe. fed MS+ 100mg/100ml	0.267±0.076
5	Leaves	0.391±0.032
6	Callus(control)	0.096±0.086

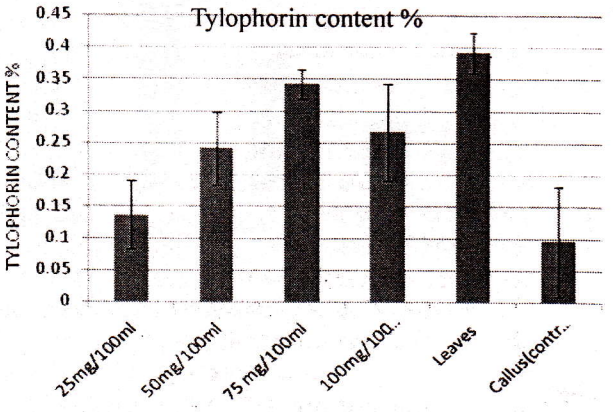


Table 2 & Fig. 3. Showing the enhanced Tylophorin content when callus grown on Zenk medium in *Tylophora indica.* Mean value ± s.e. of three replecate.

S.No.	Zenk media	Tylophorin content (%)
1	Two weeks old	0.459±0.032
2	Four weeks old	0.543±0.053
3	Six weeks old	0.696±0.087
4	Eight weeks old	0.523±0.065

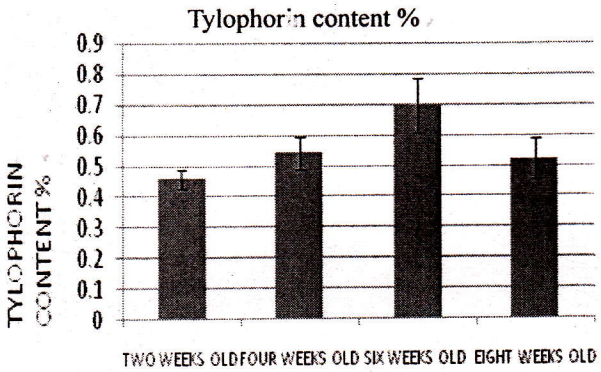
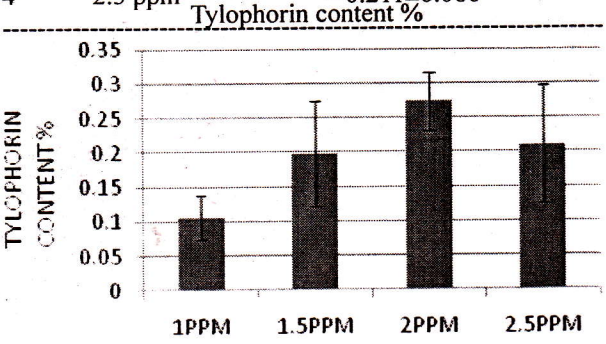


Table 3 & Fig. 4. Showing the effect of different concentration of BAP on content of tylophorin alkaloid in *Tylophora indica* tissue culture ± mean value s.e. of three replicate.

S.No.	MS+BAP	Tylophorin Content (%)
1	1ppm	0.105±0.032
2	1.5 ppm	0.198 ±0.076
3	2 ppm	0.273±0.043
4	2.5 ppm	0.211±0.086



Plants synthesize an extensive array of secondary metabolites, often with highly complex structures. Currently, most pharmaceutically important secondary metabolites are isolated from wild or cultivated plants because their chemical synthesis is not economically feasible. Biotechnological production in plant cell cultures is an attractive alternative. The production can be enhanced by using many methods like manipulation in medium, using cytokinin and also by feeding culture by a precursor[13]. In the present study tylophorin content was enhanced considerably by using these methods. In the present investigation, Zenk media showed the maximum enhancement in the tylophorin content as compared to others that may be due to the increased concentration of sucrose and lower concentration of phosophates[16] . In continuation of this work other cytokinin can be used to enhanced the tylophorin content *in vitro*. The study concluded that modification in environmental condition of plant tissue culture can work as a tool to increase the medicinally important compounds .The developed protocol has an importance in industries for commercial purpose, which is in demand.

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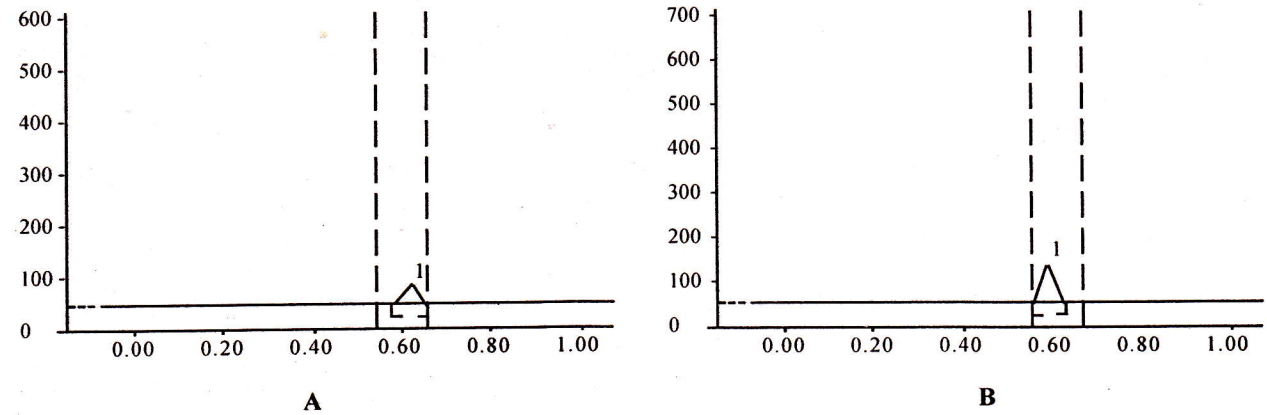

Fig.5. Showing the HPTLC analysis at 254 nm of leaves(A) with standard reference compound of tylophorin (B).

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