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BIOTECHNOLOGICAL STRATEGIES FOR ENHANCEMENT OF TYLOPHORIN IN *TYLOPHORA INDICA* (ASCLEPEADACEAE) CALLUS

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Tylophora indica , an antiasthmatic medicinal plant has been used for a long time. Here we describe a noval method for enhancement of tylophorin alkaloid in *Tylophora indica* callus. In the present study, DIPA, Kinetin, Tyrosine, Ornithine ,Salicylic acid and *Aspergillus niger* extract were used to examine their effect on tylophorin biosynthesis separately .Salicylic acid and *Aspergillus niger* extract were used in suspension culture while rest of the compound were used in static culture .Here modified Zenk media was used as the production media .The maximum enhancement in tylophorin content was obtained in Zenk media (0.82±0.05) supplemented with 60 mg/100 ml of Tyrosine with 50 G/l glucose which is almost nine fold higher than control and three fold than explant leaves. This protocol of tylophorin biosynthesis *in vitro* is not well documented till date..

Keywords : *Aspergillus niger;* DIPA; Kinetin; Ornithine; Salicylic acid; Tyrosine; Zenk media.

Introduction

The past two decades plant cell biotechnology has evolved as a promising new area within the field of biotechnology, focusing on the production of plant secondary metabolites. For most compounds of interest, *e.g.* morphine, quinine, vinblastine, atropine, scopolamine and digoxin, one has so far not been able to come to a commercially feasible process. In contrast with the production of antibiotics by micro-organisms, the plant is an already existing although not always a reliable, source of these compound Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures[1,2]. In the past years new approaches have been developed *i.e.* the culturing of differentiated cells (*e.g.* shoots, roots and hairy roots), induction of secondary metabolite production by elicitors and metabolic engineering. With the culture of differentiated cells one has in most cases been able to get production of the desired compounds in levels comparable to that of the plant; however the culture of such differentiated tissues on a large-scale in bioreactors is a major constraint. For studies of the biosynthesis, such systems are very useful[3-6].The other approach *i.e.,* the use of elicitor to increase the secondary metabolite production has been successful in several cases .However, it remains limited to a certain type of compound for each plant.

Tylophorin, an anti asthmatic and anti cancerous phenanthrro -indolizidine alkaloid[7] is the main constituent of *Tylophora indica.* It has been studied in tissue culture using different medium proportion by many scientists[8,9]. 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.*[10] for the production of indole alkaloids by *Catharanthus roseus* cells. In the present study, this strategy was taken in to consideration to enhance the production of tylophorin in *Tylophora indica* callus. Cytokinin concentration in the media also supports to enhance the secondary metabolite biosynthesis[11]. Khanna *et al.*[12] have reported the enhancement of secondary metabolite by feeding the callus with phenylalanine and cinnamic acid in various medicinal plant sps.

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, 2 ppm 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°C for 15 min).Various

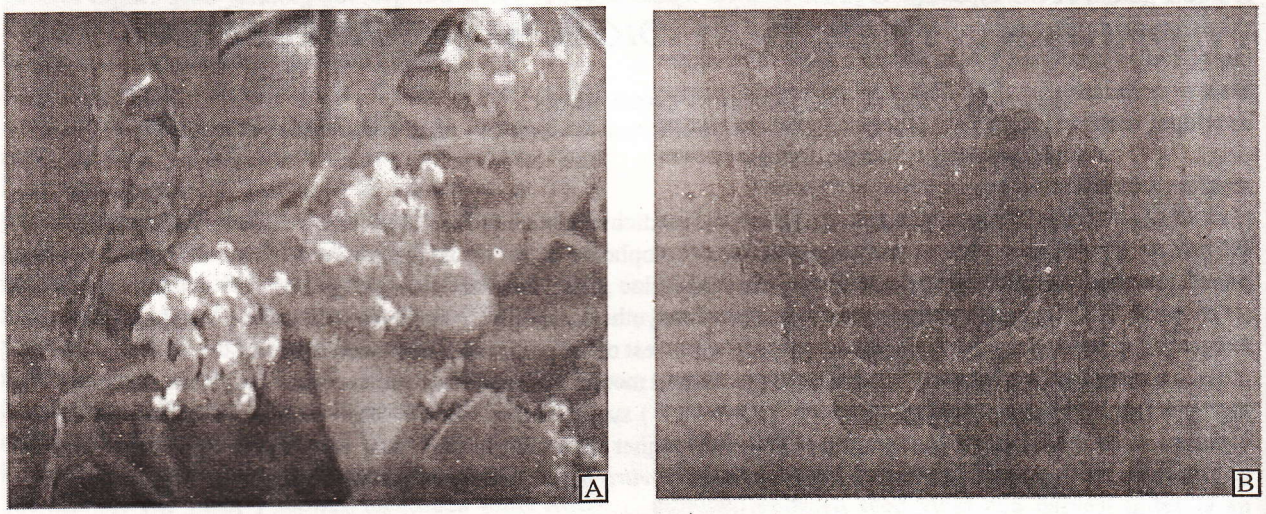

Fig.1. Showing *Tylophora.indica* plant (A) Green compact callus of *T. indica* (B).

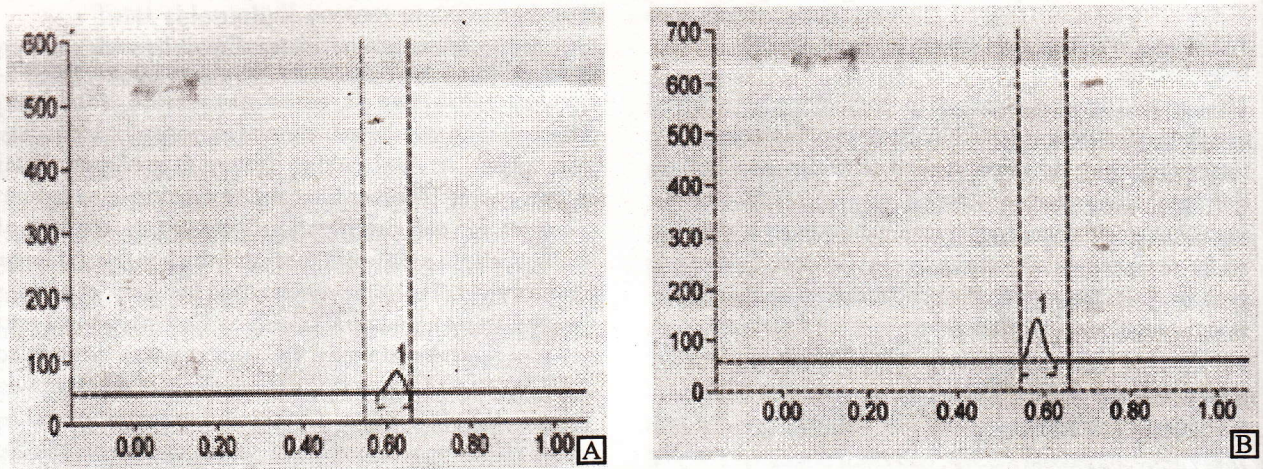

Fig .2. Showing the HPTLC analysis at 254 nm of leaves(A) with standard reference compound of tylophorin (B) in *Tylophora indica.*

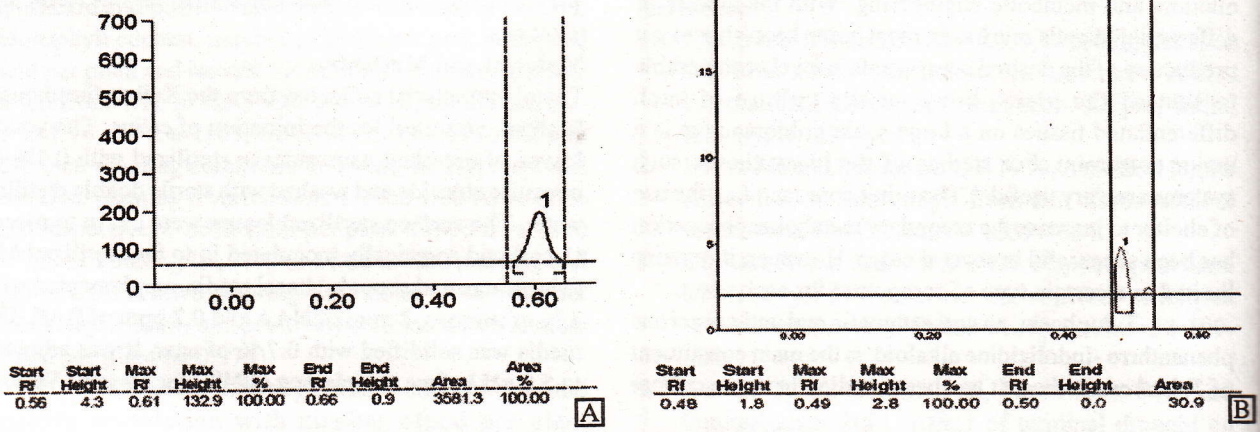

Fig.3. Showing the graphical presentation (A and B) of HPTLC analysis difference in a period of one month of tylophorin standard at 430 nm .The peak area of both the sample depicts the reduction in tylophorin accumulation.

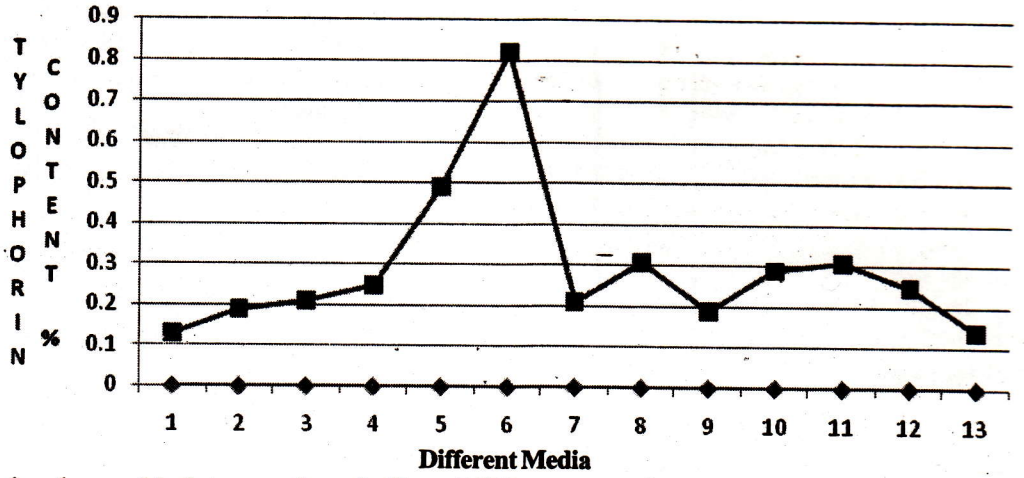

Fig.4. Showing the graphical presentation of effect of different media formulation on tylophirin production *in vitro* tissue culture of *Tylophora indica.*

Table 1. Showing the effect of different compounds on tylophorin production in tissue culture of *Tylophora indica.*

S. No.	Culture Type	Media	Compound	Sugar	Tylophorin Content (%) FW	
					Four WKS	Six WKS
1.	Callus	MS	0.1 PPM DIPA		0.09±0.122	0.13±0.043
2.	CALLUS	MS	0.2PPM DIPA		0.104±0.62	0.19±0.045
3.	CALLUS	MS	0.1KINETIN		0.15±0.321	0.21±0.034
4.	CALLUS	MS	0.2KINETIN		0.16±0. 453	0.25±0.025
5.	CALLUS	ZANK	TYROSIN 60MG/100ML		0.26±0.032	0.49±0.052
6.	CALLUS	ZANK	TYROSIN 60MG/100ML	50G/L Glucose	0.38±0.054	0.82±0.051
7.	CALLUS	ZANK	ORNITHINE40MG/100ML		0.19±0.042	0.21±0.041
8.	CALLUS	ZANK	ORNITHINE60MG/100L		0.28±0.053	0.31±0.048
9.	SUSPEN.	ZANK	SALICYCLICACID10MG/100ML		0.11±0.043	0.19±0.012
10.	SUSPEN.	ZANK	SALICYCLICACID20MG/100ML		0.21±0.064	0.29±0.045
11.	SUSPEN.	ZANK	NIL	30G/L Sucrose	0.146±0.054	0.31±0.012
12.	SUSPEN.	MS	NIL		0.19±0.012	0.25±0.041
13.	SUSPENS.	ZANK	*Aspergillus niger* 0.7mg/l	15 G/L Glucose	0.11±0.031IN 20DAYS	0.14±0.012 IN 30 DAYS
14.	CALLUS	MS	NIL	0.096±0.001		
15.	LEAVES	-	-	0.391±0.032		

other hormones like 2,4D,Kinetin ,IAA ,IBA in different combination were also used, but 2 ppm of NAA and 0.2 ppm of BAP gave the best results. The cultures were incubated at 25 -27 ^{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. The developed callus was then transferred to various media *i.e.* MS media(with 0.1,0.2 ppm of DIPA and 0.1,0.2 ppm Kinetin) and Zenk production media (with 40 and 60 mg/100 ml of Tyrosine, 40 and 60 mg/100 ml Ornithine,10 and 20 mg/100ml of Salicylic acid and 0.7% *Aspergillus niger* extract) without growth hormone separately. Leaves and all the experimental callus samples were dried, powdered, weighed and subjected for cold extraction using methanol. Thin Layer Chromatography (TLC) 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 spot 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 K GaA), Sample application was carried out on CAMAG Linomat 5 Instrument (CAMAG Linomat 5 "Linomat5_080222" S/N 080222). All the analysis were carried out using established condition.(Inert gas -Spray gas ;. Sample solvent type - Methanol; Dosage speed -150nl/s ; Syringe size -100µl ; Analysis wave length - 430 nm) Toluene: Ethyl acetate: Diethyl amine (14:2:2) was used as mobile phase.

Results and Discussion

Alkaloids are cyclic nitrogen containing secondary metabolites[13],where the nitrogen atom is derived directly from an amino acid[14]. Alkaloids are biosynthesized by many unique metabolic pathways and displayed a broad spectrum of pharmacological activities[15]. Secondary metabolites are produced in small amount where enzymatic and genetic evidence exist for these pathway[16]. The 60% of drugs from plant origin are alkaloids[17]. In the present study, the obtained callus was compact and green in color. Twenty four months maintained callus was used for the further experiments. Various compound including biotic and abiotic elicitor (DIPA, Kinetin, Tyrosine, Ornithine ,Salicyclic acid, *Aspergillus niger* extract) were taken into consideration for enhancing the tylophorin content *in vitro* tissue culture of *Tylophora indica*. Maximum tylophorin production was obtained in static culture with Zank media supplemented by 50G glucose and 60 mg/100ml of tyrosine (0.82% FW; Control 0.096)) amongst all test samples used (Table 1) which was ten fold higher than control and around tree fold higher than leaves. According to established rule[1,2] the production of secondary metabolite in suspension culture is higher than the static culture ,but in the present study, it was observed that tylophorin production was lower in suspension culture than the static culture. The exuded tylophorin could not be collected .This might be due to the oxidation of indolilizidine alkaloid[18,19]. Figure 3 also support this finding ,in which the characteristic standard tylophorin (methanolic liquid form) peak in HPTLC analysis becomes consequently smaller in time interval. So it can be said that in soluble form tylophorin is not stable and it got oxidize. Hence suspension culture is not appropriate for the tylophorin production.

Hence, tylophorin, an antiasthamatic,anti inflammatory and anticancerous alkaloid in *Tylophora indica* has been enhanced amazingly *in vitro* with the help of Plant tissue culture technique. The developed protocol is not well documented till date. The results support our previous findings[11]. Developed protocol can be immensely used by the biotech industries. Thus we can save our medicinal plants belt and consequently our ecosystem.

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