www.ijpbs.net

INTERNATIONAL JOURNAL OF PHARMA AND BIO SCIENCES

Internationally indexed journal

Indexed in Chemical Abstract Services (USA), Index coppernicus, Ulrichs Directory of Periodicals, Google scholar, CABI ,DOAJ , PSOAR, EBSCO , Open J gate , Proquest , SCOPUS , EMBASE ,etc.

Rapid and Easy Publishing

The "International Journal of Pharma and Bio Sciences" (IJPBS) is an international journal in English published quarterly. The aim of IJPBS is to publish peer reviewed research and review articles rapidly without delay in the developing field of pharmaceutical and biological sciences

Pharmaceutical Sciences

- Pharmaceutics
- Novel drug delivery system
- Nanotechnology
- Pharmacology
- Pharmacognosy
- Analytical chemistry
- Pharmacy practice
- Pharmacogenomics
- Polymer sciences
- Biomaterial sciences
- Medicinal chemistry
- Natural chemistry
- Biotechnology
- Pharmacoinformatics
- Biopharmaceutics

Biological Sciences

- Biochemistry
- Biotechnology
- Bioinformatics
- Cell biology
- Microbiology
- Molecular biology
- Neurobiology
- Cytology
- Pathology
- Immunobiology

Indexed in Elsevier Bibliographic Database

(Scopus and EMBASE)

SCImago Journal Rank 0.288

Impact factor 5.121*

ISSN 6299 -0975

Chemical Abstracts Service (www.cas.org)

A division of the American Chemical Society

CODEN IJPBJ2

Google Scholar BETA

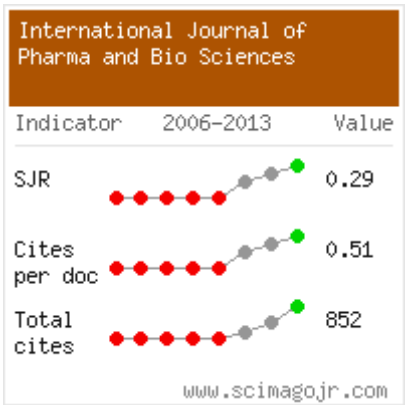
Elsevier Bibliographic databases (Scopus & Embase)

SNIP value – 0.77

SJR - 0.288

IPP - 0.479

SNIP – Source normalised impact per paper
SJR – SCImago Journal rank
IPP –Impact per publication
Source –www.journalmetrics.com
(Powered by scopus (ELSEVIER)



ProQuest

LUND UNIVERSITY

JSU JACKSONVILLE STATE UNIVERSITY

Jacksonville State University
Houston Cole Library
USA (Alabama)

UNIVERSITY OF OXFORD

Oxford, United Kingdom

INDEX COPERNICUS INTERNATIONAL

And indexed/catalogued in many more university

*Instruction to Authors visit **www.ijpbs.net**

For any Queries, visit "contact" of www.ijpbs.net

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CONSERVATION AND ENHANCED TYLOPHORINE THROUGH IN VITRO PROPAGATION AND PRECURSOR FEEDING IN *TYLOPHORA INDICA*-AN ENDANGERED MEDICINAL PLANT

KSHIPRA SONI, SUMIT SAHNI, M. Z. ABDIN AND ALKA NARULA*

Department of Biotechnology, Faculty of Science, Hamdard University, New Delhi, India

ABSTRACT

The aim of the present study was to enhance the level of tylophorine in *Tylophora indica* through micropropagation and precursor feeding. Leaf and nodal segments were inoculated on Murashige and Skoog medium supplemented with different combinations and concentrations of auxins and cytokinins to standardize the micropropagation protocol. Out of 48 concentrations tried, the best regeneration medium for node was MS+0.5mg/l Indole-3-acetic acid (IAA)+2mg/l Kinetin (Kn) and for leaf was MS+0.5mg/l IAA+1mg/l Benzyladenine (BA). 5.64-fold higher level of tylophorine was obtained in in vitro raised plant as compared to in vivo grown mother plant. Tyrosine, a precursor of tylophorine was supplemented in various concentrations for different time intervals. The highest tylophorine production was (27.71µg/g DW) obtained in presence of tyrosine (2mg/l) for 48 hrs. This level was 2.81-fold higher than control cultures and 5.87-fold higher than in in vivo grown plants. This optimized protocol can be utilized for commercial level production of tylophorine.

KEY WORDS: Tylophorine, tyrosine, secondary metabolite, precursor, micropropagation

ALKA NARULA
Department of Biotechnology, Faculty of Science, Hamdard University, New Delhi, India

***Corresponding author**

INTRODUCTION

Tylophora indica (Burm.F) Merill, commonly known as Indian Ipecac or Antamul belongs to Asclepiadaceae. It is traditionally used asa folk remedy in India for the treatment of bronchial asthma, inflammation[1,2], allergy and dermatitis[3]. The active constituents of *T. indica* are phenanthroindolizidine alkaloids (0.2- 0.46%). Total alkaloid content varies in different parts of plant, highest being in leaves (0.12-0.50%) followed by stem (0.07-0.37%) and roots (0.09-0.35%)[4]. Out of all, Tylophorine is the major alkaloid and other alkaloids identified are tylophorinine, tylophorinidine, septicine, isotylocrebrine, and tylophoricine[5]. Tylophorine and its analogues exhibit potent growth-inhibitory activity against a broad range of human cancer cells[6].

Increased demand of *T. indica* is still met through an indiscriminate collection of plants from natural habitats. Over exploitation and lack of organized cultivation led to rapid decline in the wild population of *T. indica* and resulted in its enlistment as one of the plant species endangered in India. Therefore, there is an urgent need for large scale multiplication of this plant for its conservation as well as to meet the burgeoning demand. Moreover, plants growing in the wild suffer from various climatic and environmental fluxes which bring changes in their chemical profile.

Plant tissue cultures have been perceived as promising choice for obtaining valuable chemicals throughout the year and conservation of important medicinal plants. Most important factor in micropropagation is the selection of plant growth regulators which not only control different physiological responses but also regulate the production of plant secondary metabolites[7]. Micropropagation protocols for *T. indica* have been standardized earlier by some workers[2, 8-13] but there are only two reports on the estimation of tylophorine in micropropagated plants[12, 14] and they have not compared the level of tylophorine in in vivo mother plant and tissue culture raised plantlets. Besides, the addition of plant growth regulators, there are some other methods also available for enhancement in the level of secondary metabolites such as screening of high-producing cell line, in situ product removal, immobilization, biotic and abiotic stresses, elicitation and precursor feeding. Among these, precursor feeding is one of the unique techniques to over express genes involved in thebiosynthesis pathway which stimulated the production of secondary metabolites[15] in *Rauvolfia tetraphylla*[16], *Cistanche salsa*[17], *Vitis vinifera*[18], *Mitragyna speciosa*[19] and *Withania somnifera*[20]. Tyrosine has been suggested as the precursors of the tylophorine[21]. There is no prior report on the enhancement of tylophorine yield through precursor feeding. Therefore, the major objective of the study was to establish micropropagation protocol for the conservation and obtain higher levels of tylophorine from *T. indica* via precursor (tyrosine) feeding.

MATERIALS AND METHODS

Plant material and sterilization of explants

Leaves and nodal segments of *T. indica* were collected from herbal garden of Hamdard University, New Delhi. Explants (leaf and nodal segment) were washed under running tap water and then treated sequentially with 0.2% Cetrimide (Leaf-5min., node-7min.), 0.25% Streptomycin sulphate, 0.5% Bavistin (leaf-15min., node-20 min.), 0.1% Mercuric chloride (Leaf-3min., node-5min.), 70% Ethanol (1 min. for both) and washing with sterile water (6 times).

Media and culture conditions

The sterilized explants were inoculated on MS[22] medium. The pH of media was adjusted to 5.69 prior to the addition of 0.63% (w/v) agar and autoclaved at 121°C, 15 psi for 20 min. Various combinations of IAA/NAA and BA/Kn were tried to standardize the regeneration protocol. MS basal medium served as control. The cultures were maintained in the culture room at 25±2°C with relative humidity at 55±5% and 16/8 h light and dark photoperiods. The morphogenic response was monitored periodically. During different stages of growth and differentiation (callus/ shoots/plantlets) the cultures were harvested for the secondary metabolite analysis.

Rooting and Hardening

The in vitro regenerated shoots of *T. indica* were excised and inoculated on MS medium supplemented with different concentrations of NAA and IBA (0.5/2/5mg/l) for rooting. Rooted shoots were transferred to MS basal medium. Thereafter, plantlets with well-developed thick roots were transferred to pots containing equal amount of autoclaved soilrite and soil (1:1). Pots were covered with polyethylene bags to maintain 80–90% humidity and irrigated with ½ strength MS salt solution and later on with sterile distilled water. After four weeks covers were withdrawn and hardened plants were shifted to pots with garden soil.

Precursor Feeding

Leaf derived calli (6-week-old) were used as explant for the precursor feeding experiment. Liquid MS medium containing IAA (0.5mg/l) + BA (1mg/l) was prepared and after autoclaving filter sterilized tyrosine was added at different concentrations (C-0; T1-0.5; T2-2; T3-5; T4-10 mg/l). Calli were inoculated on the above media and kept in the incubator shaker at 25°C and 100 rpm for different time intervals (24, 48 and 72 hrs). After that calli were harvested and dried in oven at 50°C for tylophorine estimation.

Tylophorine Estimation

Extraction of tylophorine[23]

0.5g (DW) samples were defatted with petroleum ether (40-60°C) in a soxhlet apparatus for 24 hrs. 25 ml chloroform was added to the defatted powder (sample) and kept in an incubator shaker at 25°C and 100 rpm for 24 hrs. Extract was filtered through whatman filter paper. This step was repeated three times. Extracts were combined and evaporated to dryness at room temperature and resuspended in chloroform.

Estimation by HPTLC analysis

High Performance Thin Layer Chromatography (HPTLC) of chloroform extract was performed using CAMAG, Switzerland. Plant extract was applied with 100µl syringe on pre coated silica gel 60 HPTLC plates (10 x 10 cm) after activation at 120°C with band length of 6 mm and track separation of 10 mm using Linomat V applicating device. The chromatograph was developed in twin trough chamber using a solvent system of chloroform:methanol:ethyl acetate (90:5:5) and scanned in scanner III at 254 nm wavelength using lamp in absorption mode. Tylophorine was detected on the basis of R_f value and spiking with a standard of tylophorine (Enzo life sciences, USA). For quantitative analysis, peak areas were used to calculate the amount of tylophorine present in the tissues, and these were compared with the standard. The standard sample was used to construct a calibration graph. Stock solution for standard was prepared by dissolving 0.5mg standard in 0.5 ml of chloroform and further dilutions were made.

RESULTS AND DISCUSSION

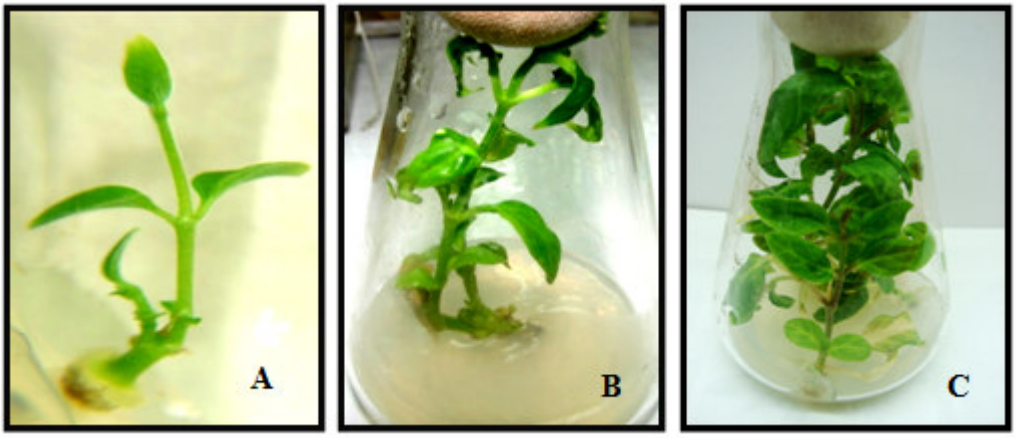
On MS media supplemented with combinations of auxins and cytokinins, regeneration was observed indirectly from leaf and directly from node. In the present study, nodal segments proved better explants for shoot regeneration over leaf. There are very few reports available on micropropagation of *T. indica* using nodes as explant. High frequency shoot regeneration was reported in presence of NAA, BA and ascorbic acid from nodes of *T. indica*[24, 25]. But in other reports ascorbic acid was not used[9,13]. In the present study, shoot induction was very poor in all the concentration of NAA and BA tried. The best results (percent shoot regeneration, number and length of shoot per explant) were obtained in presence IAA and Kn supplemented medium. IAA at 0.5mg/l has given good response with all concentrations of Kn (0.5 mg/l to 5mg/l) tried (Fig.1). Out of them IAA (0.5mg/l) with Kn (2mg/l) resulted in 100% shoot regeneration with3.59 shoots of 8.74cm/explant differentiated after 12 weeks (Table. 1). As the level of IAA increased the shoot differentiation declined. Thus, the present investigation is the first report achieving 100% shoot regeneration from nodal segment in presence of IAA and Kn containing medium. Similarly a combination of IAA and Kn was successfully used for shoot regeneration from nodal segment of *Dioscorea bulbifera*[26].

Table 1
Interactive effect of IAA and Kn on shoot regeneration from nodal segments after 12 weeks

MS+(in mg/l)	Percent Shoot regeneration	Average number of shoot per explant	Average length of shoot per explant (cm)
IAA(0.5)+Kn(0.5)	81.7±1.424^{cd}	2.00±0.090^{cde}	6.88±0.169^{c}
IAA(0.5)+Kn(1)	90.3±0.936^{b}	2.31±0.083^{c}	7.63±0.101^{b}
IAA(0.5)+Kn(2)	100±0.000^{a}	3.59±0.118^{a}	8.74±0.113^{a}
IAA(0.5)+Kn(5)	91.6±0.850^{b}	2.86±0.109^{b}	7.1±0.147^{c}
IAA(1)+Kn(0.5)	70.5±0.471^{e}	1.52±0.047^{fg}	5.53±0.128^{f}
IAA(1)+Kn(1)	78.9±0.709^{d}	1.78±0.087^{ef}	6.33±0.092^{de}
IAA(1)+Kn(2)	84.9±0.732^{c}	2.15±0.111^{cd}	6.87±0.141^{c}
IAA(1)+Kn(5)	71.5±0.776^{e}	1.88±0.117^{def}	6.14±0.158^{e}
IAA(2)+Kn(0.5)	60.2±1.421^{g}	1.06±0.103^{h}	5.38±0.133^{f}
IAA(2)+Kn(1)	65.8±0.566^{f}	1.29±0.071^{gh}	6.31±0.154^{de}
IAA(2)+Kn(2)	71.2±1.016^{e}	1.57±0.106^{fg}	6.68±0.135^{cd}
IAA(2)+Kn(5)	63.3±0.897^{fg}	1.24±0.070^{gh}	5.84±0.160^{ef}
Basal	30.1±1.352^{h}	0.25±0.050^{i}	0.38±0.075^{g}

The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same column, significant differences at the P≤0.5 level are indicated by different letters.

Figure 1
Shoot regeneration in presence of MS+IAA (0.5mg/l)+Kn (2mg/l) from nodal segment after: A.6 weeks B. 12 weeks C. 20 weeks



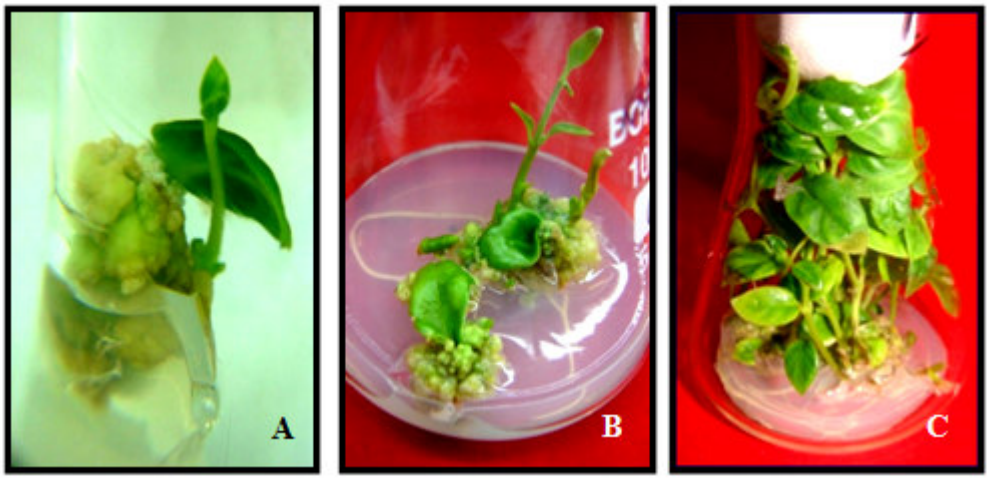
From leaf explant, callus initiated along the margins within four weeks in the presence of IAA along with BA thus, proved best combination of plant growth regulators tried. The percentage of cultures showing callusing and amount of callus increased with age (Fig. 2). After four weeks, compact green regenerative callus was induced in 80% cultures and reached 100% after 12 weeks in presence of IAA (0.5mg/l) and BA (1mg/l) (Table 2). Similarly, highest callusing was reported in presence of IAA with BA in *Ajuga bracteosa* and *Leucas aspera*[27, 28]. In contrast, different phytohormones have also been used for the induction of callus from leaf explant in *T. indica* including 2, 4, 5-T[29], 2,4-D with BA[11,30] and NAA along with BA[12]. For shoot regeneration from leaf explant of *T. indica*, BA[12, 31]/ Kinetin[10, 29]/ TDZ[30] proved effective in previous reports. However, in the present study 95.7% shoot regeneration with 3.43 shoots of 4.31 cm differentiated per explant in presence of IAA (0.5mg/l) and BA (1mg/l). Similarly, highest percent shoot regeneration and number of shoot from leaf explants of *Bacopa monnieri* in presence of IAA and BA was obtained[32].

Table 2
Interactive effect of IAA and BA on shoot regeneration from leaf explant after 12 weeks

MS+(in mg/l)	Percent callusing	Percent Shoot regeneration	Average number of shoot per explant	Average length of shoot/ per explant (cm)
IAA(0.5)+BA(0.5)	85.1±1.086^{b}	82.2±0.613^{cd}	2.49±0.073^{c}	3.52±0.090^{b}
IAA(0.5)+BA(1)	100±0.000^{a}	95.7±1.142^{a}	3.43±0.057^{a}	4.31±0.093^{a}
IAA(0.5)+BA(2)	80.7±0.759^{c}	85.7±0.873^{b}	2.96±0.081^{b}	2.88±0.050^{c}
IAA(0.5)+BA(5)	73.3±0.919^{d}	82.6±0.896^{cd}	2.75±0.050^{b}	2.60±0.077^{d}
IAA(1)+BA(0.5)	81.0±0.707^{c}	70.7±0.957^{g}	2.03±0.074^{efg}	2.43±0.065^{de}
IAA(1)+BA(1)	83.3±0.720^{bc}	84.8±0.885^{bc}	2.89±0.052^{b}	3.33±0.052^{b}
IAA(1)+BA(2)	72.1±0.936^{d}	79.8±0.792^{de}	2.45±0.071^{c}	2.24±0.045^{ef}
IAA(1)+BA(5)	63.5±0.668^{e}	77.2±0.759^{e}	2.16±0.086^{de}	1.89±0.071^{g}
IAA(2)+BA(0.5)	65.3±0.648^{e}	70.9±0.521^{fg}	1.81±0.057^{g}	2.19±0.090^{ef}
IAA(2)+BA(1)	73.5±0.677^{d}	77.9±0.638^{e}	2.31±0.088^{cd}	2.87±0.100^{c}
IAA(2)+BA(2)	58.8±1.633^{f}	73.7±0.712^{f}	2.00±0.078^{def}	2.06±0.118^{fg}
IAA(2)+BA(5)	51.1±0.638^{g}	68.7±0.824^{g}	1.84±0.064^{fg}	1.44±0.057^{h}
Basal	00.0±0.000^{h}	00.0±0.000^{h}	0.00±0.000^{h}	0.00±0.000^{i}

The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same column, significant differences at the P≤0.5 level are indicated by different letters.

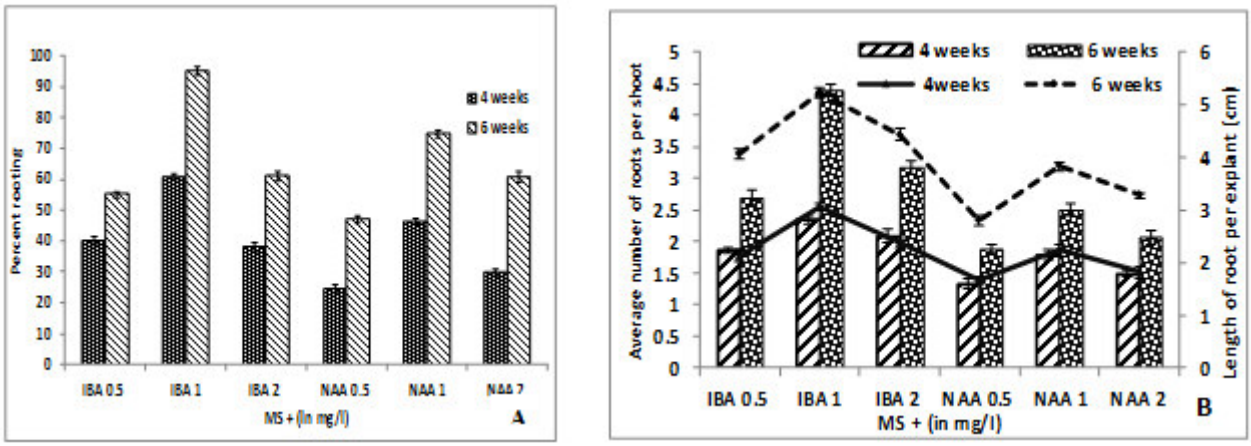
Figure 2
Shoot regeneration in presence of MS+IAA (0.5mg/l)+BA (1mg/l) from leaf after: A. 6 weeks B. 12 weeks C. 20 weeks



Rooting

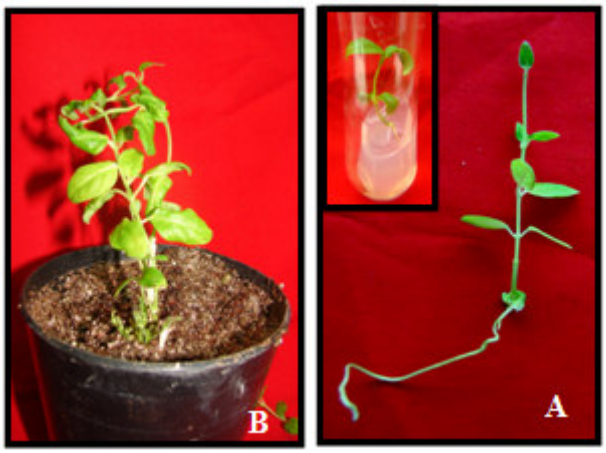
Isolated shoots were allowed to grow on IBA/NAA supplemented medium for rooting showed 95.1% rooting in presence of IBA (1mg/l) whereas only 74.7 percent shoots rooted in presence of NAA (1mg/l) after six weeks (Fig. 3). A linear increase in percentage rooting, number and length of roots per shoot was noticed up to IBA at 1mgl/1 (Fig. 4). Further increase in concentration of IBA resulted in thin roots which became yellowish after three weeks. In previous reports IBA proved as best root inducing hormone in *T. indica*[11]. However, in other studies rooting was obtained in presence of IAA[24, 31]. The possible reason for IBA to be the most suited auxin in the present work could be its resistance to chemical degradation in tissue culture media, both during autoclaving and at room temperature[33]. Rooted plantlets were hardened, acclimatized and successfully established in field conditions with 90% survival.

Figure 3
Comparative analysis of different concentrations of IBA and NAA on rooting



The data were statistically analyzed using Duncan's multiple range test. Error bars represents mean±standard error.

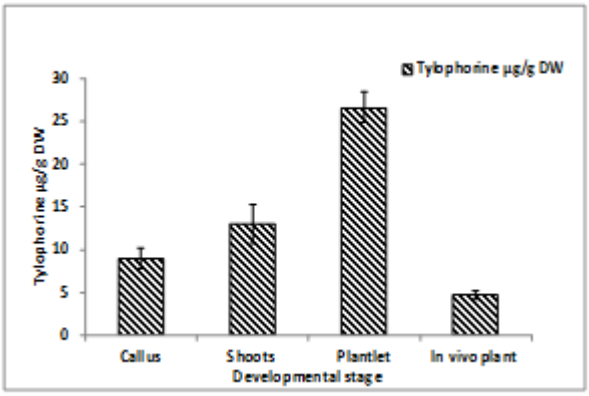
Figure 4
A. Rooting in presence of MS+IBA (1 mg/l)
B. Hardening of in vitro raised plant



Enhanced yield of tylophorine through organogenesis

Level of tylophorine was estimated in in vivo grown mother plant and at different stages of in vitro differentiation i.e. callus, shoots and plantlet. All the stages of in vitro differentiation showed higher amount of tylophorine than in vivo grown plant. Level of tylophorine increased when the differentiation progressed. Callus showed 1.89-fold enhancement which reached to 2.75-fold in shoots and maximum in plantlet (5.64-fold) when compared to mother plant (Fig. 5). Tylophorine level measured in indirectly and directly regenerated plants of *T. indica* was 80 and 71µg/ml[14] respectively. Later on same group of workers[12] showed maximum tylophorine (80µg/ml) in the leaves of in vitro regenerated plants, followed by suspension (28.30µg/ml) and callus cultures (24.46µg/ml). The present study is the first report showing a comparison of tylophorine content among different stages of in vitro differentiation and in vivo source plant. Similarly higher yield of secondary metabolites was reported in in vitro raised tissue as compared to field grown plant of *D. bulbifera*[26], *B. monnieri*[34] and *Cichorium intybus*[35].

Figure 5
Tylophorine in different stages of in vitro plant development (Source of explant- node) and in vivo grown plant

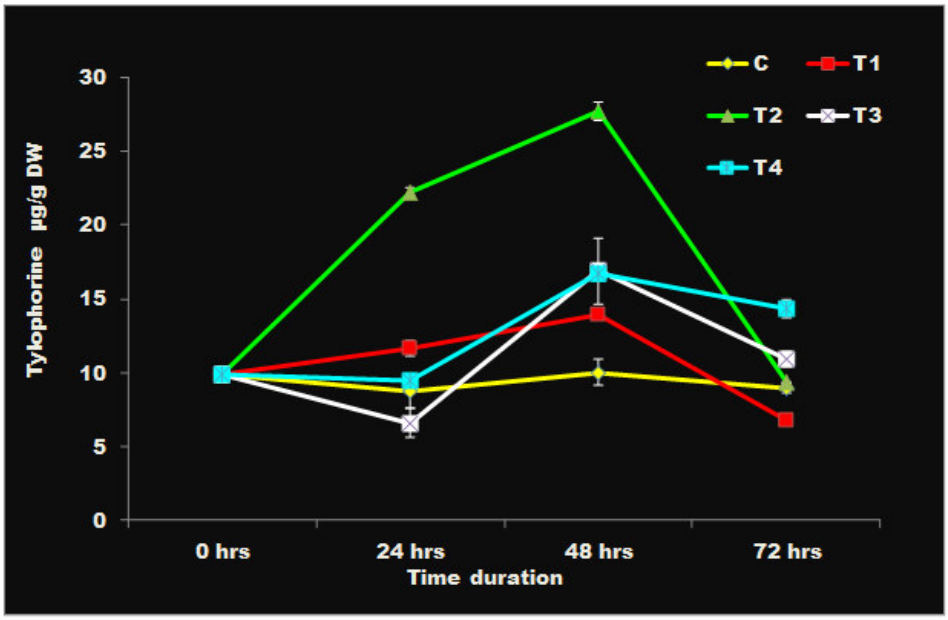


The data were statistically analyzed using Duncan's multiple range test. Error bars represents mean±standard error.

Enhanced yield of tylophorine through precursor feeding

Precursor feeding to in vitro cultures is useful for enhancing the biosynthesis of secondary metabolites. To best of author's knowledge there is no earlier report on enhanced tylophorine by feeding of precursor in *T. indica*. The data revealed that the most suitable concentration and time duration for the highest tylophorine production (27.71µg/g DW) was 2mg/l tyrosine for 48hrs. This level was 2.81-fold higher than control cultures and 5.87-fold higher than in vivo grown plant. Beyond 2mg/l tylophorine level declined. With increasing the time duration of treatment tylophorine level increased up to 48 hrs and if fed for longer time the level of tylophorine decreased (Fig. 6). Similarly 554% more taxifoline was obtained in *Silybum marianum* after feeding coniferyl alcohol for 48 hrs[36]. Recently other study also recommends 48 hrs as optimum feeding time for increasing the yield of rosmarinic acid in *Solenostemon scutellariodes*[37]. 23% more flavonoid was obtained in *Hydrocotyle bonariensis* by feeding 3mg/l phenylalanine[38] and 2.35-fold and 2.04-fold higher 1, 2-didehydrostemofoline and stemofoline in *Stemona* sp was observed after treatment with precursor sodium acetate at 25 mg/l[39].

Figure 6
Effect of precursor feeding on the level of Tylophorine



The data were statistically analyzed using Duncan's multiple range test. Error bars represents mean±standard error.

CONCLUSION

The standardized protocol can be used for large scale propagation of *T. indica* for its conservation as well as for the enhanced level of tylophorine. Higher level of tylophorine i.e. 5.64 and 5.87-fold can be obtained in 20-week-old plantlets and in 44-day-old callus through feeding of tyrosine at 2mg/l for 48hrs over the in vivo grown plants respectively.

ACKNOWLEDGEMENT

Ms. Kshipra Kapil (née Soni) express sincere thanks to Department of Science and Technology (WOS-A scheme) for financial support.

REFERENCES

1. Gopal Krishnan C, Shankarnarayan D, Kameswaran L and Natarajan S, Pharmacological investigation of tylophorine. Indian J Med Res, 69: 513-520, (1979).
2. Faisal M, Singh S and Anis M, In vitro regeneration and plant establishment of *Tylophora indica*: petiole callus culture. In vitro Cell Dev Biol-Plant, 41: 511-515, (2005).
3. Gore KV, Rao AK and Guruswamy MN, Physiological studies with *Tylophora asthmatica* in bronchial asthma. Indian J Med Res, 71: 144-148, (1980).
4. Karnick CR, Phytochemical investigations of some *Tylophora* species found in India. Planta Med, 27: 333-336, (1975).
5. Gupta M, Mukhatr HM and Ahmad S, Phyto-pharmacological and plant tissue culture overview of *Tylophora indica* (burm f.) Merill. J Pharm Sci Res, 2: 401-411, (2010).
6. Wu CM, Yang CW, Lee YZ, Chuang TH, Wu PL, Chao YS and Lee SJ, Tylophorine arrests carcinoma cells at G1 phase by down regulating cyclin A2 expression. Biochem Biophys Res Commun, 386: 140-145, (2009).
7. Sharmila R, Subburathinam KM and Sugumar P, Effect of growth regulators on andrographolide production in callus cultures of *Andrographis paniculata*. Adv Biotechol, 12: l7-20, (2013).
8. Thomas TD, Isolation, callus formation and plantlet regeneration from mesophyll protoplast of *Tylophora indica*. In Vitro Cell Dev Biol-Plant, 45: 591-598, (2009).
9. Kaushik A, Gurnani C, Sunder S, Dhingra A and Chimpa V, Biochemical assessment of in vitro and in vivo culture of *Tylophora indica* (Burm. f.) Merr. Kathmandu Univ J Sci, Eng Technol, 6: 1-5, (2010).
10. Rathinavel S and Sellathurai T, In vitro regeneration and phytochemical screening of *Tylophora indica*, an endangered medicinal herb. J Exp Sci, 1: 4-6, (2010).
11. Sadguna V, Swamy TN, Raju S, Ghani M, Suresh V and Mustafa M, High frequency regeneration of plantlets from leaf derived callus cultures of *Tylophora indica* Burmf. An important medicinal plant. Int J Sci Eng Res, 4: 2704-2707, (2013).
12. Kaur H, Anand M and Goyal D, HPTLC based analysis of tylophorine from cultures and in vitro regenerated plants of *Tylophora indica*- an endangered medicinal plant. Int J Pharma Res Sch, 3: 91-95, (2014).
13. Rajavel L and Stephan R, Low cost in vitro propagation of *Tylophora indica* (Burm f.) Merrill. using different carbon sources. J Acad Ind Res, 3: 221-224, (2014).
14. Kaur H, Anand M and Goya D, Establishment of efficient protocol for micropropagation of stem explants of *Tylophora indica*, an important medicinal plant. Afr J Biotechnol, 10: 6928-6932, (2011).
15. Varnisree M and Hsin-Seng T, Plant Cell Cultures- An alternative and efficient source for the production of biologically important secondary metabolites. Int J Appl Sci Eng, 2: 29-48, (2004).
16. Anitha S and Kumari BDR, Stimulation of reserpine biosynthesis in the callus of *Rauvolfia tetraphylla* L. by precursor

feeding. Afr J Biotechnol, 5: 659-661, (2006).
17. Liu J, Guo Z and Zeng Z, Improved accumulation of phenylethanoid glycosides by precursor feeding to suspension culture of *Cistanche salsa*. Biochem Eng J, 33: 88-93, (2007).
18. Saw NMMT, Riedel H, Kütük O, Ravichandran K and Smetansaka I, Effect of elicitors and precursors on the synthesis of anthocyanin in grape (*Vitis vinifera*) cell cultures. Energy Res J, 1: 189-191, (2010).
19. Zuldin NNM, Said IM, Noor NM, Zainal Z, Kiat CJ and Ismail I, Induction and analysis of the alkaloid mitragynine content of a Mitragyna speciosa suspension culture system upon elicitation and precursor feeding. The Sci World J, Article ID 209434: 11 pages, (2013).
20. Sivanandhan G, Selvaraj N, Ganapathi A and Manickavasagam M, Enhanced biosynthesis of withanolides by elicitation and precursor feeding in cell suspension culture of Withania somnifera (L.) Dunal in shake-flask culture and bioreactor. PLoS ONE, 9: e104005, (2014).
21. Bhakuni DS and Mangla VK, Biosynthesis of tylophorine and tylophorinine. Tetrahedron, 37: 401-407, (1981).
22. Murashige T and Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant, 15: 473-497, (1962).
23. Chaudhuri KN, Ghosh B, Tepfer D and Jha S, Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: growth and tylophorine productivity in different transformed root clones. Plant Cell Rep, 24: 25-35, (2005).
24. Sharma N and Chandel KPS, Effect of ascorbic acid on axillary shoot induction in *Tylophora indica* (Burm. F.) Merril. Plant Cell Tiss Org Cult, 29: 109-113, (1992).
25. Faisal M, Ahmad N and Anis M, An efficient micropropagation system for *Tylophora indica:* an endangered, medicinally important plant. Plant Biotechnol Rep, 1: 155-161, (2007).
26. Narula A, Kumar S, and Srivastava PS, Genetic fidelity of in vitro regenerants, encapsulation of shoot tips and high diosgenin content in *Dioscorea bulbifera* L. a potential alternative source of diosgenin. Biotechnol Lett, 29: 623-629, (2007).
27. Kaul S, Das S and Srivastava PS, Micropropagation of *Ajuga bracteosa*, a medicinal herb. Physiol Mol Biol Plants, 19: 289-296, (2013).
28. Dharishini MP, Balasubramanian K and Radha A, In vitro micropropagation of *Bacopa monnieri* and detection of bacosides from secondary callus. J Acad Ind Res, 3: 233-236, (2014).
29. Faisal M and Anis M, Rapid mass propagation of *Tylophora indica* Merrill via leaf callus culture. Plant Cell Tiss Org Cult, 75: 125-129, (2003).
30. Thomas TD and Philip B, Thidiazuron induced high frequency shoot organogenesis from leaf derived callus of a medicinal climber, *Tylophora indica* (Burm.f.) Merrill. In Vitro Cell Dev Biol-Plant, 41: 124-128, (2005).
31. Bera TK and Roy SC, Micropropagation of *Tylophora indica* (Burm. f.) Merrill. by multiple bud formation from mature leaf explants without callus intervention. Bot Bull Acad Sci, 34: 83-87, (1993).
32. Binita BC, Ashok MD and Yogesh TJ, *Bacopa monnieri* (L.) Pennell: A rapid, efficient and cost effective micropropagation. Plant Tissue Cult Biotechnol, 15: 167-175, (2005).
33. Hussein S, Ling APK, Hann NG T, Ibrahim R and Paek KY, Adventitious roots induction of recalcitrant tropical woody plant, *Eurycoma longifolia*. Romanian Biotechnolog Lett, 17: 7026-7035, (2005).
34. Jain M, Rajput and Anamika M, Enhancement of secondary metabolite biosynthesis in *Bacopa monnieri*: an in vitro study. Res J Recent Sci, 2: 13-16, (2013).
35. Ali Z, Ganie SH, Narula A, Abdin MZ and Srivastava PS, Efficient regeneration and Esculin content in *Cichorium intybus* L., a potent medicinal plant. International Journal of Pharma and Bio Sciences 4:1333-1340, (2013).

36. Tumova L, Rimakova J, Tuma J and Dusck J, *Silybum marianum* in vitro – flavolignan production. Plant Cell Environ, 52: 454-458, (2006).
37. Dewanjee S, Gangopadhyay M, Das U, Sahua R and Khanra R, Enhanced rosmarinic acid biosynthesis in *Solenostemon scutellarioides* culture: a precursor-feeding strategy. Nat Prod Res, 28: 1691-1698, (2014).
38. Masoumian M, Arbakariya A, Syahida A and Maziah M, Effect of precursors on flavonoid production by *Hydrocotyle bonariensis* callus tissues. Afr J Biotechnol, 10: 6021-6029, (2011).
39. Dheeranupattana S and Chaichana N, Effects of sodium acetate and sucrose on in vitro alkaloid production from *Stemona* sp. culture. Asian J Plant Sci, 12: 92-96, (2013).