



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

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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 (27.71 μ g/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



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INTRODUCTION

Tylophora indica (Burm.F) Merrill, commonly known as Indian Ipecac or Antamul belongs to Asclepiadaceae. It is traditionally used as a folk remedy in India for the treatment of bronchial asthma, inflammation^{1,2}, allergy and dermatitis³. 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, isotylocrine, and tylophoricine⁵. Tylophorine and its analogues exhibit potent growth-inhibitory activity against a broad range of human cancer cells⁶.

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 metabolites⁷. 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 the biosynthesis pathway which stimulated the production of secondary metabolites¹⁵ 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 tylophorine²¹. 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 MS²² 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 tylophorine²³**

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

applying 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

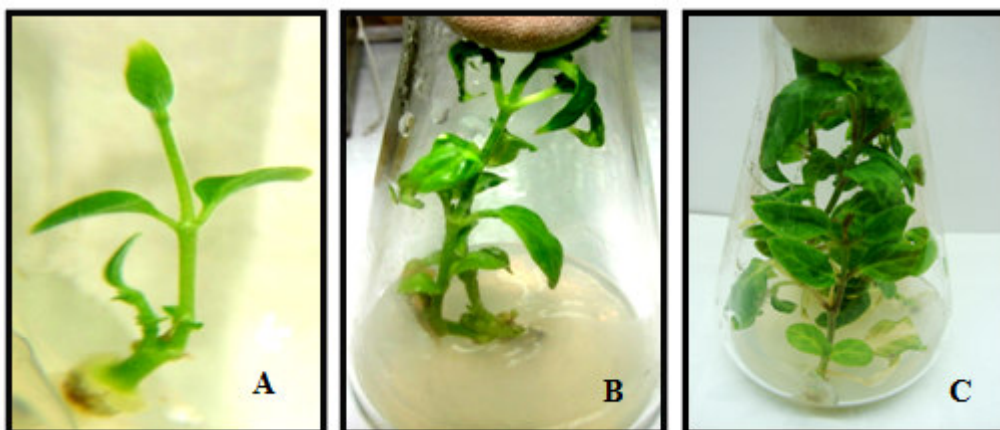
On MS media supplemented with combinations of auxins and cytokinins, regeneration was observed indirectly from leaf and directly from node. In the present study, nodal segments proved better explants for shoot regeneration over leaf. There are very few reports available on micropropagation of *T. indica* 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 with 3.59 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 ^c	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 ^c	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 ^c	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-T²⁹, 2,4-D with BA^{11,30} and NAA along with BA¹². For shoot regeneration from leaf explant of *T. indica*, BA^{12, 31}/ Kinetin^{10, 29}/ TDZ³⁰ 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 obtained³².

Table 2

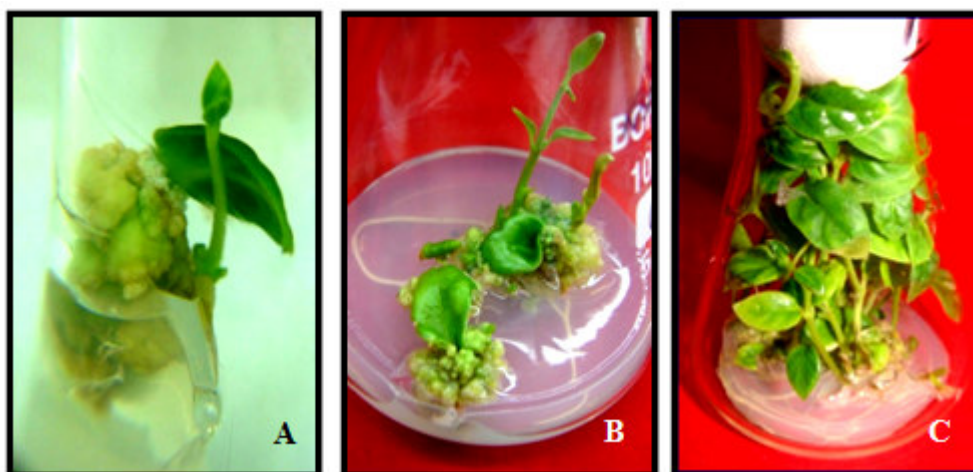
Interactive effect of IAA and BA on shoot regeneration from leaf explant after 12 weeks

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 ^l

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

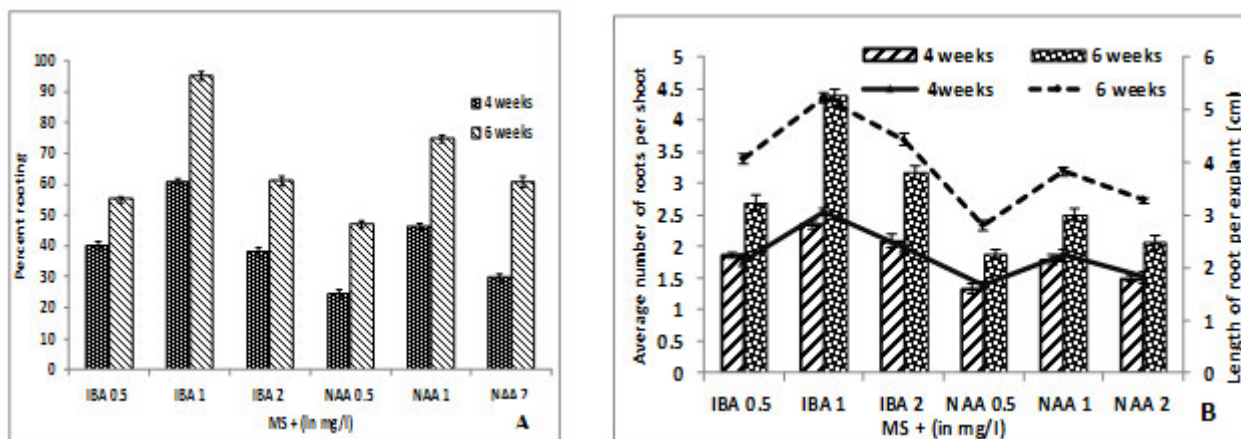


Rooting

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 1mg/l (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 temperature³³. Rooted plantlets were hardened, acclimatized and successfully established in field conditions with 90% survival.

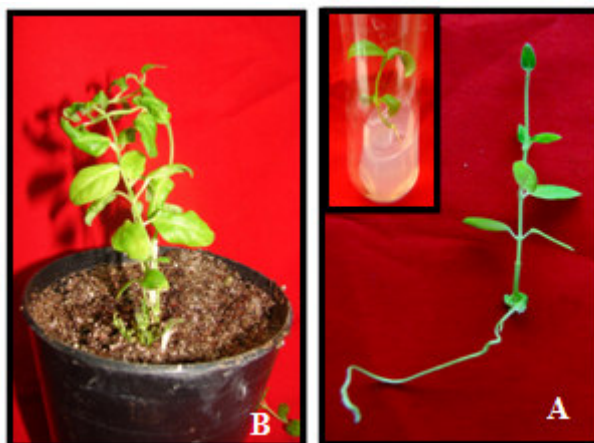
Figure 3

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

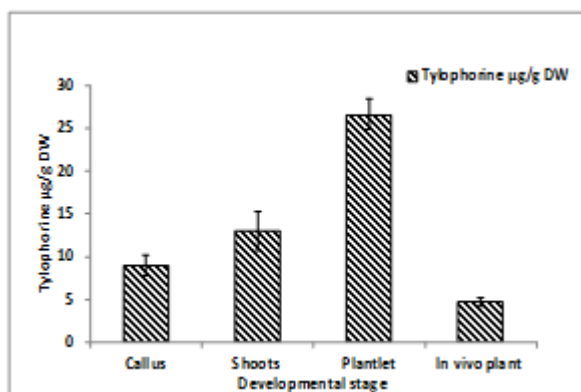


Enhanced yield of tylophorine through organogenesis

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 71 $\mu\text{g/ml}$ ¹⁴ respectively. Later on same group of workers¹² showed maximum tylophorine (80 $\mu\text{g/ml}$) in the leaves of in vitro regenerated plants, followed by suspension (28.30 $\mu\text{g/ml}$) and callus cultures (24.46 $\mu\text{g/ml}$). 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. monnier*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



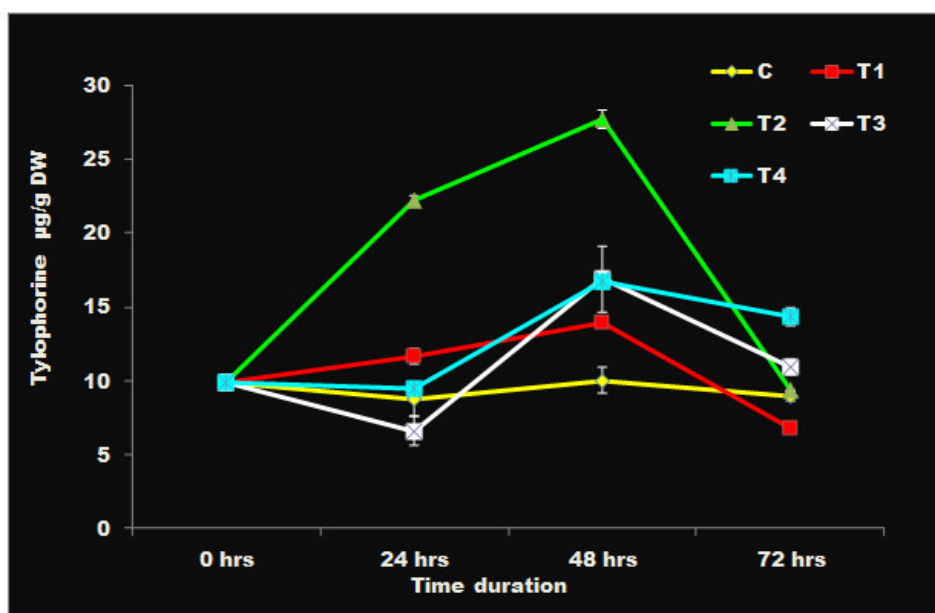
The data were statistically analyzed using Duncan's multiple range test. Error bars represents mean±standard error.

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 (27.71 µg/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 hrs³⁶. Recently other study also recommends 48 hrs as optimum feeding time for increasing the yield of rosmarinic acid in *Solenostemon scutellarioides*³⁷. 23% more flavonoid was obtained in *Hydrocotyle bonariensis* by feeding 3mg/l phenylalanine³⁸ 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/l³⁹.

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.

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