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RESEARCH ARTICLE

Standardized micropropagation protocol for *DAMVEL* [*Tylophora indica*] via indirect organogenesis

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Abstract

Traditional propagation of *Tylophora indica* is challenging due to low seed viability, poor germination and difficulty in rooting vegetative cuttings. Micropropagation offers the best solution for rapid and true-to-type plant multiplication. The study aims to standardize a micropropagation protocol in *Tylophora indica* (Burm. F.) Merrill through indirect organogenesis from leaf explant. This protocol includes sterilization of leaf explant, callus production, shoot regeneration, root initiation and acclimatization. Surface sterilization treatment using 70 % ethanol for 2 min, 0.1 % bavistin for 15 min, 1000 ppm streptomycin for 10 min and 0.1 % $HgCl_2$ for 3 min found the most effective. The best callus, which was dark green, friable and heaviest (3.53 g) was observed in MS media supplemented with 1 mg L^{-1} Benzyl Adenine (BA). MS media supplemented with 2 mg L^{-1} BA showed the highest shoot regeneration (65.94 %) and maximum number of shoots per callus (12.90). *In vitro* regenerated plantlets were inoculated into MS media supplemented with different concentrations of Indole-3-acetic acid (IAA). MS media supplemented with 0.5 mg L^{-1} IAA was found to be the most effective for rooting (83.33 %) and with the longest roots (7.60 cm). *In vitro* rooted plantlets were acclimatized successfully after primary and secondary hardening. Primary hardening mixture, which was a mixture of cocopeat and soil in a 1:2 ratio, resulted in 80 % survival rate. After secondary hardening, the overall plant survival rate was 75 %.

Keywords: *in vitro*; micropropagation; multiple shooting; rooting; *T. indica*

Introduction

Tylophora indica (Burm. F.) Merrill (*Apocynaceae*), commonly known as Indian ipecac or Antamul, is traditionally known to treat asthma and hence is also known as 'asthma herb' (1).

The genus *Tylophora* comprises 60 species that are mainly distributed in tropical and subtropical Asia, Africa and Australia. *T. indica* is an important threatened medicinal plant commonly known as Antmul. Medically *T. indica* is an important plant and very efficiently used in all the systems of medicine. It is a perennial, small, slender, much branched, pubescent, twining or climbing herb found in the sub-Himalayan tract from Uttar Pradesh to Meghalaya and in central and peninsular India. It has also been reported from Eastern, North-East and Central India, Bengal and parts of South India (2).

This indigenous medicinal plant has been traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, bronchitis, rheumatic pain and dermatitis. Root of the plant has been often employed as an effective substitute for ipecac. *T. indica* has been reported to contain 0.46 % of alkaloids (3). The main active constituents of the plant are the alkaloids tylophorine and tylophorinine. Also, the root contains a potential anti-tumor alkaloid tylophorinidine. The root and leaves possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic properties and are used for the treatment of asthma, bronchitis, whooping cough, dysentery and diarrhoea. They are also effective in rheumatic and gouty pains and hydrophobia (4).

Because of the shortage of high quality planting material, commercial cultivation of *Damvel* is uncommon and wild populations are exploited for the extraction of secondary metabolites. Due to the lack of adequate propagation efforts and overexploitation of natural wild populations, it is threatened with extinction and listed as one of the endangered species. It is therefore imperative to adopt alternative methods having high multiplication rates to produce large number of plants of improved quality and shortened rotation. To obtain *Tylophora* planting materials that are true to type, a micropropagation approach may be helpful in this regard (5). *In vitro* regeneration of *T. indica* has been reported using various explants such as axillary buds, roots, leaves and stem/petiole segments (6-13). However, these protocols often exhibit low multiplication rates, limited rooting efficiency and poor acclimatization, with minimal emphasis on survival under field conditions.

Furthermore, a reproducible and standardized micropropagation protocol employing diverse explants and optimized growth regulator combinations remains underdeveloped. Considering the species' endangered status

and overexploitation in the wild, the present study was undertaken to establish an efficient protocol for indirect organogenesis and rooting to support large-scale propagation, conservation and sustainable utilization of *T. indica*.

This process makes it possible to produce more healthy plants, which promotes the species' growth, spread and raises its biodiversity. *T. indica* must be cultivated *in vitro* in order to be used commercially and preserved. Therefore, the research work was carried out on *Damvel.*

Materials and Methods

Preparation of explants materials and establishment of culture

Leaves of *T. indica* were used as an explant for indirect organogenesis. Healthy and young leaves were collected from the Botanical Garden, Pari Talav, Junagadh Agricultural University, Junagadh in October-November, 2023. Small pieces of leaves were inoculated *in vitro* for the experiment.

Surface sterilization of leaf explant

Inside the laboratory, disease free, young and healthy leaves were washed under running tap water for 20 min. After being soaked in water that contains 0.1 % Bavistin with 1-2 drops of Tween 20. To remove any residual chemicals, explants were rinsed thoroughly with distilled water, repeating this process 3-4 times.

Under aseptic conditions in laminar air flow, four different sterilizations were used in this experiment. Treatments which were given are coded in Table 1. After the treatment of $HgCl_2$, explants were washed with autoclaved water (3-4 times) for the removal of residual chemicals.

Since $HgCl_2$ is toxic, all procedures involving it were conducted in a fume hood with appropriate PPE and all waste was collected in labelled hazardous waste containers and disposed of according to institutional biosafety and chemical waste disposal protocols.

Inoculation into growth media

Following sterilization, the next step was the preparation and inoculation of explants into suitable culture media. Leaf explant was cut into small pieces (1 cm^2) and ensured that each segment contained midrib portion (Fig. 1). Small square portion of leaf explant was inoculated into MS media with different concentrations of BA (1.0-5.0 mgL^{-1}) and TDZ (0.05-2.0 mgL^{-1}) with the help of sterile forceps. Cultures were kept in the dark for four days and then exposed to culture room maintained at 25 ± 2 ºC, 40 % to 60 % relative humidity and 10/14 hr light/dark regime photoperiod.

Sub-culturing and shoot multiplication

To ensure continuous proliferation and multiplication, sub-culturing was performed at regular intervals. For the leaf explant, sub culture was done at 35 to 40 days. Callus clumps were divided into 2-3 sub-clumps and small injuries were done with help of scalpel and then transferred to fresh media in respective media of different concentrations of BA (B_1-B_5) (1.0-5.0 mgL^{-1}) and TDZ (T_1-T_5) (0.05-2.0 mgL^{-1}) for further multiplication. Subsequently, well-developed shoots were shifted to rooting media.

Table 1. Treatment inside LAF

Sterilant	Duration			
	ST1	ST2	ST3	ST4
0.4 % Bleach	15 min	20 min	-	-
70 % Ethanol	-	-	1 min	2 min
Autoclaved water wash	1 min	1 min	1 min	1 min
0.1 % Bavistin	10 min	15 min	10 min	15 min
1000 ppm Streptomycin	10 min	10 min	10 min	10 min
0.1 % HgCl2	3 min	3 min	3 min	3 min
Autoclaved water wash (3-4 times)	5 min	5 min	5 min	5 min

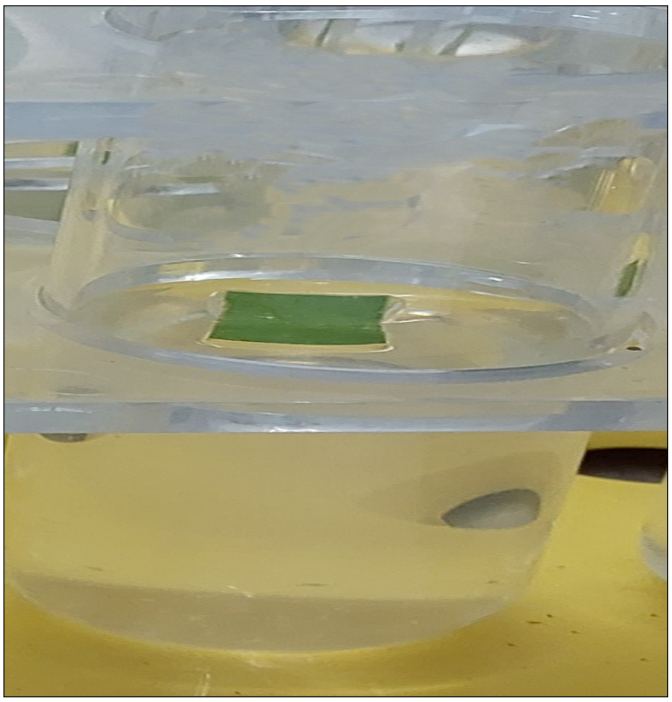

Fig. 1. Inoculation of leaf explant.

Rooting of *in vitro* multiplied shoot

The next stage involved rooting of the elongated *in vitro* shoots. Shoots that attained a length of 5.0-5.5 cm after 30 days of multiplication were carefully transferred to MS solid media enriched with IAA at varying concentrations (0.5-2.5 mgL^{-1}) to promote elongation and root formation.

Hardening and acclimatization

Once rooting was achieved, the plantlets were gradually acclimatized to external environmental conditions. Healthy *in vitro* grown rooted shoots were gently washed with tap water to remove residues of media. Then rooted plantlets were dipped into Bavistin solution to avoid infection. Then plantlets were acclimatized in greenhouse with two different phases.

Primary hardening was done in plastic cups with three different hardening mixtures of cocopeat : soil (1:2), cocopeat : sand (1:1) and vermiculite : perlite (2:1). Plastic cups were covered with another plastic cup to maintain moisture. After an interval of 2-3 days holes were made to maintain aeration.

After 15 days of primary hardening, successfully acclimatized and healthy plantlets were transferred to polythene bags containing a potting mixture of sterile soil and sand in a 3:1 ratio for secondary hardening, allowing them to establish in more natural conditions before final transfer to the field.

Statistical analysis

Statistical analysis of data of various characters will be carried out as per CRD (completely randomized design) method described in earlier research (14). The parameters of percentage shoot regeneration and percentage rooting have been transformed using the arcsine transformation. Arcsine transformation is used in percentage data when the coefficient of variation (CV) is high, indicating large variability relative to the mean. This transformation

helps stabilize the variance and normalize the distribution, making the data more suitable for parametric statistical analysis. It is especially useful when percentage values are near 0 % or 100 %, where variability tends to be inconsistent (15).

Different four surface sterilisation treatments were applied with four repetitions. Twenty-five tubes were inoculated in each repetition of four treatments. At inoculation phase of leaf explant to different growth media, twenty tubes were inoculated in each repetition of all the treatments. At the rooting stage, shoots were inoculated in six treatments along with control and each treatment was repeated four times. Total thirty-six bottles were included for rooting stage in each repetition. While total fifteen rooted plantlets were transferred in each repetition of three different hardening mixture.

Results and Discussion

Surface sterilization

Among the various treatments tested for surface sterilization for leaf explant, ST_4 [70 % ethanol for 2 min, 0.1 % Bavistin for 15 min, 1000 ppm Streptomycin for 10 min and 0.1 % $HgCl_2$ for 3 min] was found as the best treatment with the least contamination (31 %) and highest survival (69 %). Whereas, ST_1 [0.4 % bleach for 15 min, 0.1 % Bavistin for 10 min, 1000 ppm Streptomycin for 10 min and 0.1 % $HgCl_2$ for 3 min] was the least effective treatment in terms of total contamination (75 %) and total survival (25 %) (Table 2 & Fig. 2).

Indirect organogenesis from leaf explant

Leaves of *T. indica* were collected for callus production and cultured on MS media supplemented with different concentrations of BA and TDZ. Cell proliferation started within 10 -12 days. During sub-culture, callus clumps were divided. Cell proliferation was stimulated from the injured part of callus (Fig. 3). Morphological characteristics of callus were studied after 30 days of first sub-culture. BA is a cytokinin that promotes cell division and dedifferentiation, which is crucial for callus formation. Optimum BA levels stimulate rapid and organized callus growth. Higher cytokinin levels (e.g., 4-5 mgL^{-1} BA) can inhibit callus growth due to cytokinin overdose, leading to hormonal imbalance and stress. TDZ is a phenylurea cytokinin-like compound, effective even at low concentrations, but very low levels (0.05 mgL^{-1}) may be insufficient to trigger dedifferentiation (16, 17).

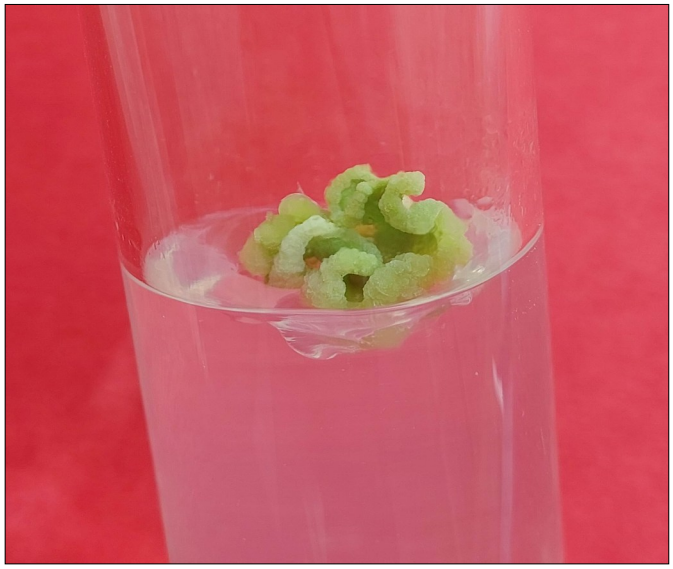

Fig. 3. Callus initiation.

Treatments	Leaf explant	
	Contamination (%)	Survival (%)
ST_1	75	25
ST_2	64	36
ST_3	52	48
ST_4	31	69
Range	31-75	25-69
Coefficient of range (%)	58.66	46.80
S.Em.±	2.12	2.12
C.D. at 5 %	6.12	6.12
C.V. %	7.64	9.53

Among all the treatments, control failed to produce callus. Excellent growth of callus was found in MS media which is supplemented with 1 mgL^{-1}BA (B_1), 2 mgL^{-1} BA (B_2) and 2 mgL^{-1} TDZ (T_5). While poor growth of callus was found in higher dose of BA [4 mgL^{-1}BA (B_4) and 5 mgL^{-1}BA (B_5)] and lower dose of TDZ 0.05 mgL^{-1} (T_1) (Table 3).

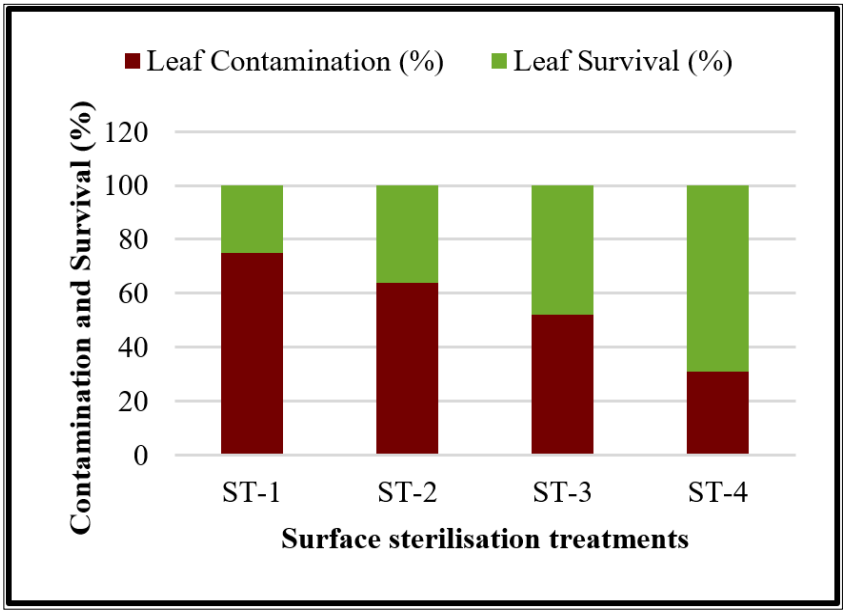

Fig. 2. Effect of various surface sterilization treatments on contamination (%) and survival (%) for leaf explant of *T. indica.*

The leaf-derived callus exhibited a range of colours i.e., light green, green, dark green and brownish. Dark green coloured calluses were found in treatment B_1 (1 mgL^{-1} BA) while light green callus found in B_3, B_4, T_2, T_3 and T_4 (Table 3).

The two types of callus tissues are common i.e., soft callus and hard callus. These can be distinguished based on their textures. Soft calluses are friable in nature and are composed of an uneven mass of cells that have little contact with one another. The hard callus, on the other hand, is compact in nature and is made up of large cells, tracheid-like cells and closely packed cells. Both kinds of callus were obtained for the current study (Table 3).

Callus weight was taken by randomly taking one callus from each treatment. Highest weight of callus was observed in MS (3.53 g) which was supplemented with 1 mgL^{-1} BA and lowest weight of callus was observed in lower dose of TDZ (0.05 mgL^{-1}) (0.91 g) (Table 3 & Fig. 4).

Friable calluses were sub cultured in respective media of BA and TDZ with four replications. The highest shoot regeneration percentage [65.94 % (83.33 %)] and the highest number of shoots per callus (12.90) were observed in MS which is supplemented with 2 mgL^{-1} BA media and the lowest percentage of frequency [24.11 % (16.67 %)] and lowest number of shoots per callus (3.50) were observed in MS with 4 mgL^{-1} BA media (Fig. 5). All other treatments except B_1, B_2, B_3 and B_4 were failed to produce shoots from callus (Table 4 & Fig. 6, 7). Shoot regeneration is heavily influenced by the cytokinin to auxin ratio. A higher cytokinin concentration (especially BA) favours organogenesis toward shoot formation. BA promotes cell division and differentiation into shoot meristems and at 2 mgL^{-1}, the concentration seems optimal for *T. indica*. Higher BA (4 mgL^{-1}) can be supra-optimal, leading to callus vitrification or abnormal shoot development (18). Results are in agreement with results obtained in earlier research (19). The highest number of shoots per callus in MS was found with 2 mgL^{-1} BA (20).

Table 3. Effect of different concentrations of BA and TDZ on leaf callus growth, colour, type and weight (g) in *T. indica*

Treatments	Callus growth	Callus color	Callus type	Callus weight (g)
Control (MS media)	-	-	-	-
B_1 (MS + 1 mgL^{-1} BA)	++++	Dark green	Friable	3.53
B_2 (MS + 2 mgL^{-1} BA)	++++	Green	Friable	2.54
B_3 (MS + 3 mgL^{-1} BA)	++	Light green	Compact	2.41
B_4 (MS + 4 mgL^{-1} BA)	+	Light green	Compact	1.37
B_5 (MS + 5 mgL^{-1} BA)	+	Green + brownish	Compact	1.53
T_1 (MS + 0.05 mgL^{-1} TDZ)	+	Brownish	Compact	0.91
T_2 (MS + 0.5 mgL^{-1} TDZ)	++	Light green	Friable	2.01
T_3 (MS + 0.1 mgL^{-1} TDZ)	+++	Light green	Friable	2.27
T_4 (MS + 1 mgL^{-1} TDZ)	+++	Light green	Friable	3.17
T_5 (MS + 2 mgL^{-1} TDZ)	++++	Yellowish green	Friable	2.43

Callus growth

- : No callus
\+ : Poor
++ : Moderate
+++ : Good

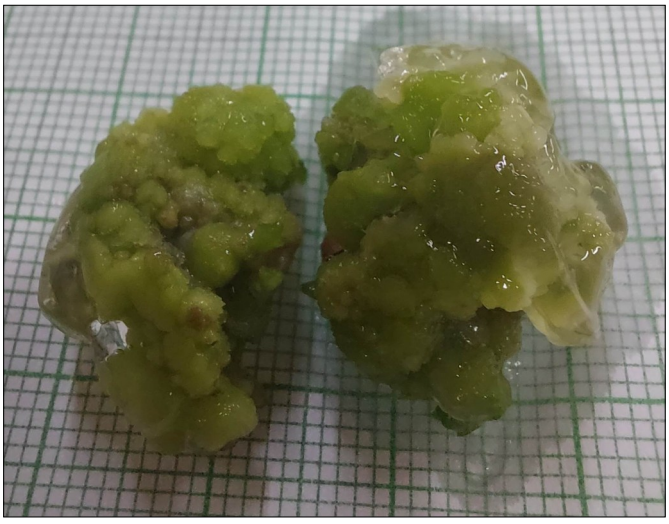

Fig. 4. Callus structure and colour in 1 mgL-1 BA.

Rooting of *in vitro* multiplied shoot

In vitro regenerated shoots were inoculated into MS media supplemented with different concentrations of IAA (0.5-2.5 mgL^{-1}) for *in vitro* rooting of *T. indica*. For root induction, 0.5 mgL^{-1} IAA was the most effective. It initiated roots in an average of 11.83 days, with 83.33 % rooting success and the longest root length of

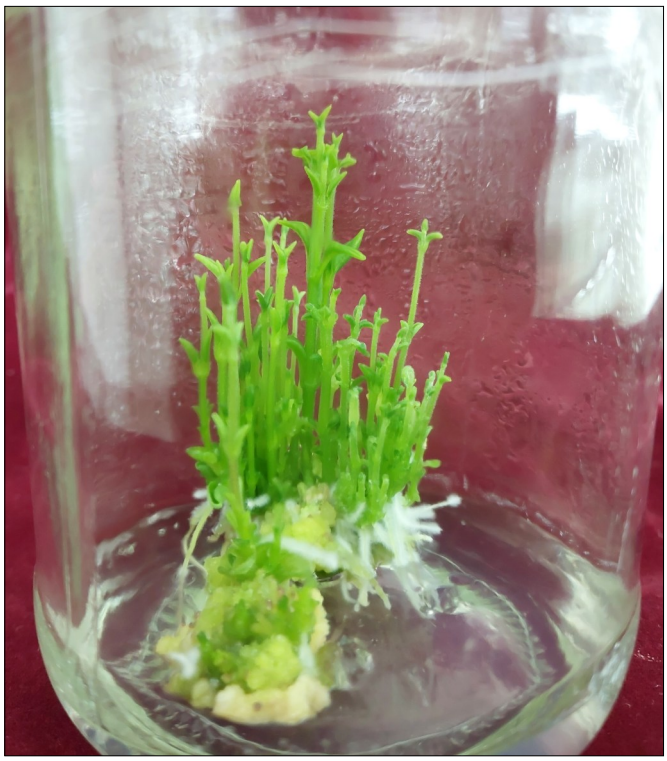

Fig. 5. Shoots regeneration in 2 mg L^{-1}.

Table 4. Effect of different concentrations of BA and TDZ on shoot regeneration (%) and number of shoots per callus of *T. indica*

Treatments	Shoot regeneration (%)	Number of shoots per callus
B_1 (MS + 1 mgL^{-1} BA)	54.76 (66.67)	8.30
B_2 (MS + 2 mgL^{-1} BA)	65.94 (83.33)	12.90
B_3 (MS + 3 mgL^{-1} BA)	32.49 (29.17)	5.10
B_4 (MS + 4 mgL^{-1} BA)	24.11 (16.67)	3.50
B_5 (MS + 5 mgL^{-1} BA)	4.16 (0.00)	-
T_1 (MS + 0.05 mgL^{-1} TDZ)	4.16 (0.00)	-
T_2 (MS + 0.5 mgL^{-1} TDZ)	4.16 (0.00)	-
T_3 (MS + 0.1 mgL^{-1} TDZ)	4.16 (0.00)	-
T_4 (MS + 1 mgL^{-1} TDZ)	4.16 (0.00)	-
T_5 (MS + 2 mgL^{-1} TDZ)	4.16 (0.00)	-
Range	0.00-83.33	3.50 - 12.90
Coefficient of range (%)	100.00	40.67
S.Em.±	0.88	0.20
C.D. at 5%	2.55	0.59
C.V. %	8.74	5.48

Note: Data outside the parentheses are arcsine transformed, whereas inside are original values.

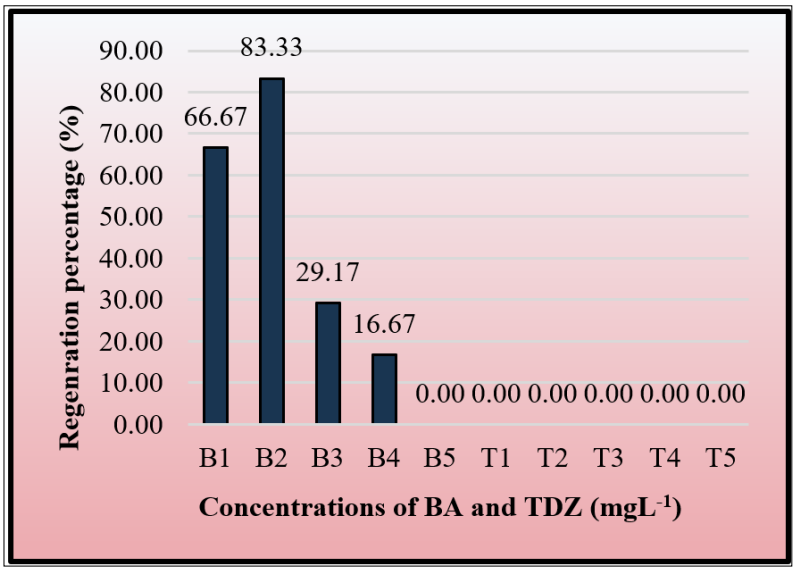

Fig. 6. Effect of various concentrations of BA and TDZ on regeneration percentage (%) of T. *indica.*

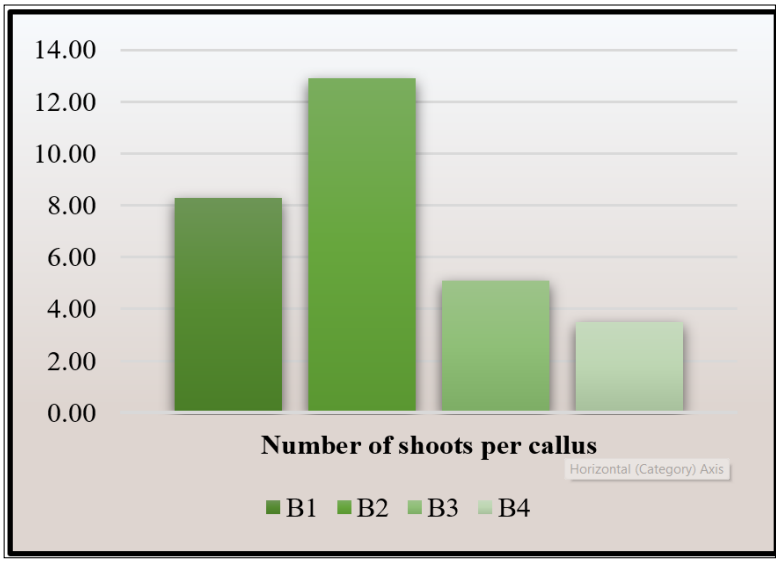

Fig. 7. Effect of various concentrations of BA on number of shoots per callus of *T. indica.*

7.60 cm (Fig. 8, 9). However, the percentage of rooting decreased as the concentration of IAA increased (Table 5 & Fig. 10, 11). IAA is a natural auxin essential for root initiation. Low concentrations promote early rhizogenesis by inducing expression of root primordia genes. At higher IAA levels, feedback inhibition or auxin-induced ethylene biosynthesis may suppress root elongation or reduce rooting efficiency (21, 22). The result of the rooting percentage is in agreement with the earlier reports (23).

Hardening of *in vitro* grown plantlets

Well rooted plantlets were transferred to plastic cup containing mixture of cocopeat : soil (1:2) (H_1); cocopeat : sand (1:1) (H_2); vermiculite : perlite (2:1) (H_3). Among different hardening mixture, H_1[cocopeat : soil (1:2)] composition was found more effective with 80 % survival percentage, which was at par with treatment H_3 (Fig. 12). However lowest survival percentage (40 %) found in cocopeat : sand (1:1) composition.

After secondary hardening, the overall survival rate of the plantlets was 75 % (Fig. 13). This indicated that *T. indica* can be effectively multiplied on a large scale and conserved using the described micropropagation method.

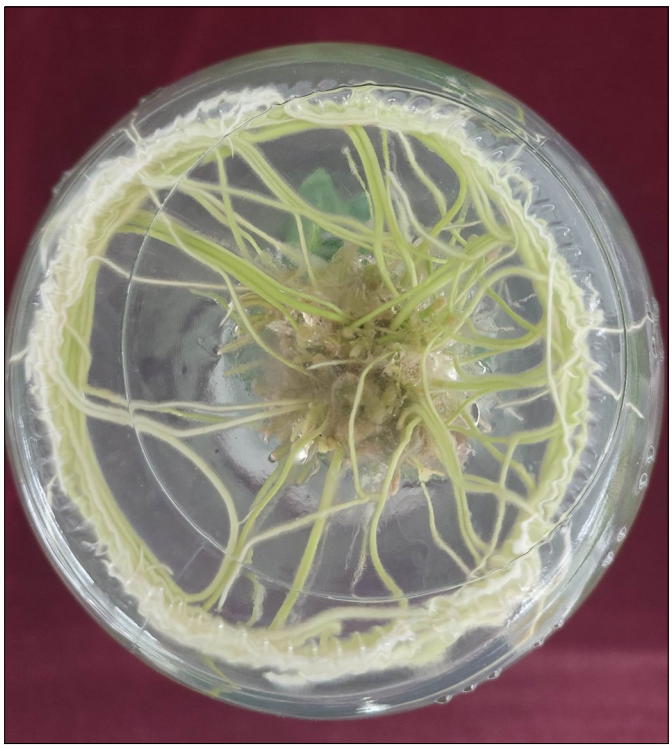

Fig. 8. Root formation in 0.5 mgL-1 IAA.

Fig. 9. Root length in 0.5 mgL-1 IAA.

Table 5. Effect of different concentrations of IAA on days to root initiation, rooting (%) and length of roots of *T. indica*

Treatments	Days to root initiation	Percentage of rooting (%)	Length of roots (cm)
Control (MS media)	14.54	54.76 (66.67)	4.41
R_1 (MS + 0.5 mgL^{-1} IAA)	11.83	65.94 (83.33)	7.60
R_2 (MS + 1 mgL^{-1} IAA)	12.67	60.35 (75.00)	5.79
R_3 (MS + 1.5 mgL^{-1} IAA)	16.58	45.02 (50.00)	4.57
R_4 (MS + 2 mgL^{-1} IAA)	19.25	40.15 (41.67)	2.93
R_5 (MS + 2.5 mgL^{-1} IAA)	20.71	35.28 (33.33)	2.52
Range	11.83-20.71	33.33-83.33	2.52-7.60
Coefficient of range (%)	27.27	42.85	50.18
S.Em.±	0.11	1.75	0.05
C.D. at 5%	0.31	5.04	0.13
C.V. %	1.34	6.95	2.01

Note: Data outside the parentheses are arcsine transformed, whereas inside are original values.

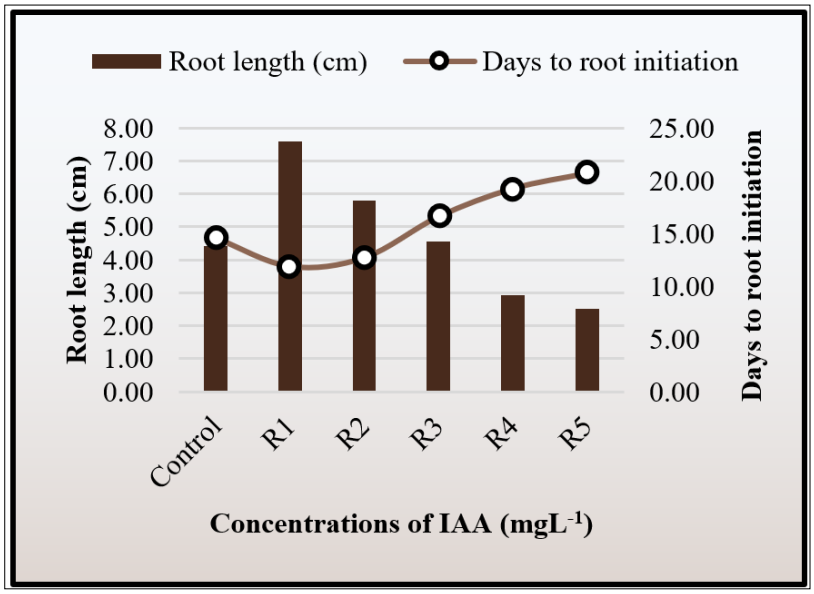

Fig. 10. Effect of various concentrations of IAA on root initiation and days to root initiation of *T. Indica.*

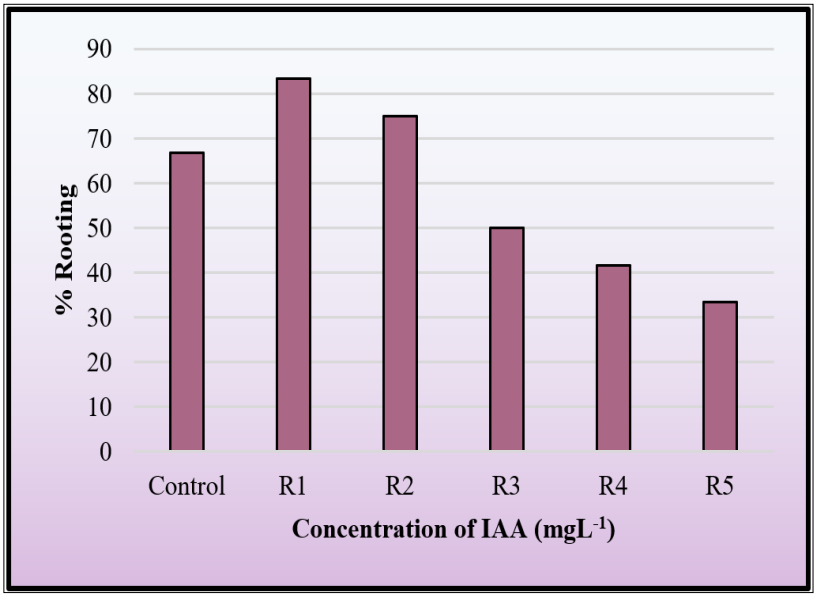

Fig. 11. Effect of various concentrations of IAA on rooting percentage (%) of *T. Indica.*

Fig. 12. Plantlets in primary hardening.

Fig. 13. Secondary hardening.

Conclusion

The study successfully established an efficient and reproducible micropropagation protocol for *T. indica* using leaf explants, demonstrating effective callus induction, shoot regeneration and rooting. The protocol proved reliable across different stages, including acclimatization, supporting its applicability for mass propagation. This *in vitro* technique offers significant potential for the conservation of this endangered medicinal plant, ensuring a sustainable supply of genetically uniform planting material. Moreover, the standardized protocol can be utilized for commercial cultivation and pharmaceutical applications, particularly in meeting the growing demand for plant-derived alkaloids with therapeutic properties.

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Authors' contributions

DBB designed and conducted the experiments, collected the data, performed statistical analyses and drafted the manuscript. VBR provided critical guidance and supervision throughout the research. DBK and MSP contributed to data collection and assisted with statistical analysis. RK and SBC supported the manuscript editing and participated in the article review process. LJR critically reviewed the manuscript and provided valuable feedback. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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