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High frequency plant production via shoot organogenesis and somatic embryogenesis from callus in *Tylophora indica*, an endangered plant species

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Abstract: An efficient and reproducible protocol has been developed for the in vitro production of an endangered medicinal climber *Tylophora indica* (Burm.f.) Merr. via leaf explants. Different types of calli produced on benzyladenine (BA) and thidiazuron (TDZ) supplemented Murashige and Skoog (MS) basal medium were selected for shoot induction and somatic embryogenesis studies. Calli when transferred from BA (5 µM) and TDZ (2.5 µM) to MS medium containing BA (5 µM) resulted in high frequency shoot induction (26.8 ± 0.97 shoots/culture) along with somatic embryogenesis (10.20 ± 0.37 embryoids/culture) up to 3 subculture passages. Embryoids transformed into complete plantlets when transferred to growth regulator free half-strength MS medium. Auxin specific pigmented and whitish roots were induced in the microshoots when planted on half-strength MS medium augmented with indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) respectively. Regenerated plantlets were hardened, acclimatised, and established in soil with 90% survival rate and exhibited normal morphology when compared with parent plants.

Key words: *Tylophora indica*, leaf callus, somatic embryogenesis, shoot organogenesis

Introduction

In vitro propagation techniques offer great potential not only for rapid multiplication of existing stock of plant species but also for conservation of important, elite, and endangered ones such as *Tylophora indica* (Burm.f.) Merr. (Faisal et al., 2007), which is a medicinal climber that belongs to the family Asclepidaceae. The leaves and roots are used medicinally against bronchial asthma, dysentery, whooping cough, bronchitis, and diarrhoea (Kirtikar & Basu, 1975; Bhavan, 1992; Varrier et al., 1994). The pharmacological activity of this plant is mainly due to the presence of alkaloid tylophorine and tylophorenine (Faisal & Anis, 2003).

Due to its overexploitation for commercial purposes and indiscriminate use, *T. indica* has become a threatened plant species facing extinction. Its poor seed germination rate also prevents its large scale propagation (Thomas & Philip, 2005). Such a situation requires the standardisation of efficient propagation methods for sustainable utilisation and management of this endangered species. Advances in

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biotechnology, particularly in plant cell tissue culture, have provided new means for conserving and rapidly propagating valuable, endangered, and rare plant species (George & Sherrington, 1984).

In vitro organogenesis leading to considerable frequency of plantlet production has already been reported through nodal segment culture (Faisal & Anis, 2006) but very few studies have been reported on induction of somatic embryogenesis in *T. indica*, which had limited success (Chaudhuri et al., 2004; Chandrasekhar et al., 2006). However, high frequency somatic embryogenesis on BA supplemented MS medium has not been reported so far.

In the present study, an efficient and cost effective protocol has been established for micropropagation of this endangered medicinal climber via indirect organogenesis and somatic embryogenesis through leaf explants on simplified nutritional and plant growth regulator supply with judicious selection of callus type.

Materials and methods

Explant preparation

Young and fully expanded leaves of *Tylophora indica* were collected from the 5-year-old climber maintained in the botanical garden of Aligarh Muslim University, India. Leaves were washed under running tap water for 30 min and then immersed in Teepol solution (5% mild detergent, v/v) for 15 min. Thereafter the leaves were washed thoroughly under running tap water. Surface sterilisation was carried out under laminar flow hood, by soaking leaves in 0.1% w/v solution of $HgCl_2$ for 3 min followed by 3-4 rinses in sterile distilled water to remove all the traces of $HgCl_2$.

Planting medium and culture conditions

Basal nutrient medium consisted of MS salts and vitamins (Murashige & Skoog, 1962) along with 3% (w/v) sucrose (Qualigens) and 0.8% (w/v) agar (Hi-media). pH of the medium was adjusted to 5.8 using HCl (1 N) or NaOH (1 N) solutions prior to autoclaving for 15 min at 121 °C and 15 lbs/in^2 pressure to decontaminate the medium of any previously present microbial contamination. After inoculation all cultures were incubated in controlled conditions with 25 ± 2 °C, 16 h light/8 h dark photoperiod, 40 $\mu Em^{-2} s^{-2}$ of light intensity provided by cool white fluorescent tubes (Philips, India Ltd.). For each set of experiment 10 replicates/concentrations were studied. All the experiments were repeated 3 times. Data were recorded periodically for any noticeable change.

Induction of organogenesis and somatic embryogenesis

The sterilised leaves were cut into 1 cm^2 pieces and inoculated on MS basal medium supplemented with a range of BA (1.0, 2.5, 5.0, 7.5, and 10 μM) and TDZ (1.0, 2.5, 5.0, 7.5, and 10 μM) concentrations individually. Yellow, friable calli were selected for study of somatic embryogenesis and the hard compact calli for shoot bud organogenesis. Subculturing of leaf derived calli on fresh medium was done after 20 days of its induction for the study of induction of shoot buds and somatic embryogenesis.

Multiplication and proliferation of shoot buds

The donor tissue was regularly subcultured after harvesting the elongated shoots for root induction, at an interval of 4 weeks on fresh medium comprised of MS plus BA (5 μM). The data were recorded after every 4 weeks of subculture.

Multiplication and germination of somatic embryos

The friable embryogenic calli produced in higher and lower concentrations of BA and TDZ respectively were transferred onto MS + BA (5 μM) for further multiplication of somatic embryoids. Data were recorded after every 4 weeks of subculturing.

Rooting in microshoots

Microshoots up to 2 to 3 cm long were excised and individually transferred to root induction medium comprising half-strength MS basal medium supplemented with various concentrations of IBA (0.1, 0.5, 2.5, and 5 μM) and NAA (0.1, 0.5, 2.5, and 5 μM). The data were recorded after 4 weeks of transfer.

Acclimatisation

The in vitro raised plantlets were transferred to thermocole (expanded polystyrene) cups containing soilrite (peat moss + perlite, 3:1) covered with transparent plastic bags to maintain about 90% RH

and were kept in controlled conditions of 55 ± 5% RH, 16 h photoperiod of 2500 lux intensity provided by 20 W fluorescent tubes (Philips, India Ltd.). The plastic bags were removed gradually after 2 weeks to expose the plantlets to the culture room environment. After 3 weeks plants were transferred to field conditions in earthen pots containing autoclaved soil plus garden manure (1:1) for proper hardening. Morphology, growth, and development of acclimatised plants were compared with those of the parent plants.

Statistical analysis

All experiments were repeated 3 times and 10 replicates per treatment were studied. Response was noted after every 3 days. The data were analysed statistically using SPSS 11 (SPSS Inc., Chicago, IL, USA). Analysis of variance was used to test the statistical significance and the significance of differences among means was tested using Tukey's test at P = 0.05.

Results and discussion

Effect of BA and TDZ on callus induction

Callus induction from the peripheral cut end and midrib region of the leaf explants was observed on BA (1.0, 2.5, 5.0, 7.5, and 10 μM) containing MS medium after 15 days of culture. Initially the callus was slow growing, friable and yellowish green. After 4 weeks, the callus turned green, hard, and compact in the middle region while the peripheral potion remained yellowish green and friable at BA (5.0 and 7.5 μM) (Figure 1A). Calli that formed at lower concentrations of BA were hard and pigmented.

In contrast, slightly loose yellowish green callus was produced from the cut end of leaf segments on (2.5 μM) TDZ supplemented MS medium after 20 days of inoculation (Figure 1B). At lower concentrations the callus was nonorganogenic and small, whereas on higher concentrations compact and nodular calli were produced.

For further studies hard, green compact calli and yellowish green, friable calli were selected for the study of shoot bud induction and somatic embryogenesis, respectively.

Effect of BA on Shoot bud induction

When the hard compact middle calli were subcultured onto different concentrations of BA, growth in callus resumed and within 4 weeks of culture nodulation in hard compact callus was started (Figure 1C). BA (5 μM) was found to be most effective for shoot bud induction wherein the nodular structures started to differentiate into an organised structure of shoot buds. These buds later transformed into healthy shoots with a maximum of 11.80 ± 0.97 shoots/culture, which is much higher than the previous report where only 9.5 shoots/culture was reported on MS+BA (10.72 μM) (Chaudhuri et al., 2004) (Table 1). The advantageous role of 5 μM BA for satisfactory shoot bud organogenesis and plantlet production was also advocated by Faisal and Anis (2003) in *Tylophora indica*, Anis and Faisal (2005) in *Psoralea corylifolia* L., and Faisal et al. (2006) in *Mucuna pruriens* (L.) DC.

The number of shoot buds differentiated decreased to 7.6 ± 0.75 shoots/culture at 10 μM of BA (Table 1). Nema et al. (2007) in *T. indica* also recorded a similar pattern of shoot bud differentiation and suggested that the higher concentration of cytokinin had an inhibitory effect on shoot bud formation and elongation.

Effect of BA on Somatic embryogenesis

Somatic embryos were induced in yellowish green friable peripheral calli when cultured at higher concentrations of BA (5 and 7.5 μM) within 3 weeks of culture (Figure-1D), while no such observation was recorded at lower concentrations (Table 1). Somatic embryos underwent germination while still attached to the mother tissue when kept for longer duration in the induction medium (Figure 2A). Embryos at different stages of development were isolated from the peripheral callus mass (Figure 2B), which was similar to the observations reported by Rao and Narayanswami (1972) in *T. indica*. Mature embryoids were transferred to hormone free MS medium for further maturation and germination (Figure 2C). So far no report is evident for somatic embryogenesis on BA supplemented MS medium in plant species. However, there are several reports of somatic embryogenesis on BA along with 2,4-D or NAA in other plant species but induction of embryogenesis on BA alone is a very rare phenomenon (Yasuda et al., 1985; Pasquale et al., 1994).

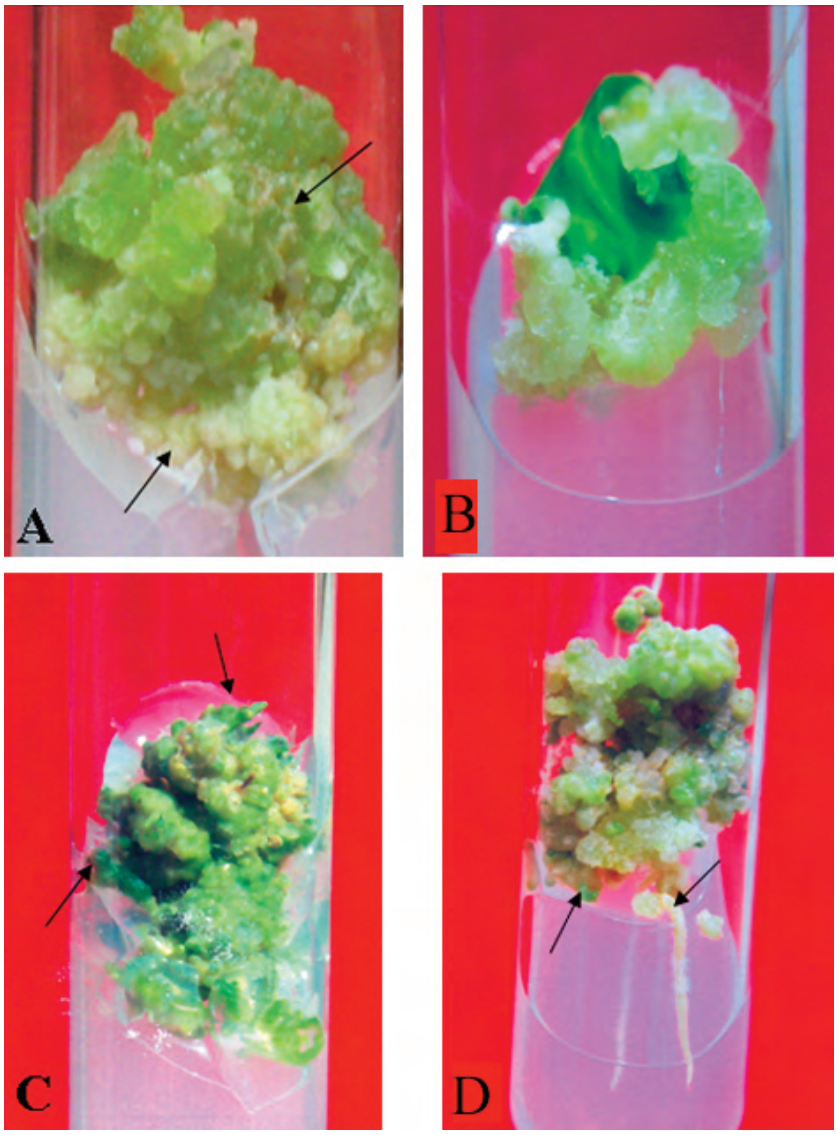

Figure 1. A) Four-week-old leaf callus induced on MS + BA (5 µM) showing compact, green middle portion with yellowish green, friable peripheral portion.
B) Leaf explant showing callus induction on MS + TDZ (2.5 µM).
C) Middle, hard, green nodular callus showing high frequency shoot bud induction when subcultured on MS + BA (5 µM).
D) Callus (5 weeks old) produced on MS + BA (5 µM) showing somatic embryogenesis in peripheral portion with middle portion showing shoot bud induction.

Effect of TDZ on callus induction and regeneration of shoots and embryoids

Yellowish green, slightly loose callus was recorded after 4 weeks of culture (Figure 3A). A lower concentration of TDZ (2.5 µM) was found to favour high frequency shoot bud induction (18.2 ± 0.58 shoots/culture) along with a low rate of somatic embryogenesis (3.8 ± 0.37 embryoids/culture) (Figure 3B), whereas poor results were obtained at a higher concentration (Table 1). Similar results were reported by Faisal and Anis (2003) in *T. indica* where TDZ (2.5 µM) showed the highest shoot regeneration frequency

Table 1. Effect of BA and TDZ on shoot regeneration and somatic embryogenesis via leaf callus culture.

BA (μM)	TDZ (μM)	Mean no. of somatic embryoids/culture	Mean no. of shoots/culture	Mean shoot length (cm)
1.0	-	0.00 ± 0.00^{c}	3.00 ± 0.32^{d}	1.56 ± 0.17^{d}
2.5	-	0.00 ± 0.00^{c}	5.00 ± 0.55^{cd}	2.70 ± 0.29^{c}
5.0	-	5.80 ± 0.37^{a}	11.8 ± 0.97^{a}	5.40 ± 0.22^{a}
7.5	-	5.00 ± 0.32^{a}	8.80 ± 0.37^{b}	4.20 ± 0.20^{b}
10.0	-	3.20 ± 0.37^{b}	7.60 ± 0.75^{bc}	4.10 ± 0.25^{b}
-	1.0	2.20 ± 0.37^{b}	7.60 ± 0.75^{c}	3.40 ± 0.28^{b}
-	2.5	3.80 ± 0.37^{a}	18.20 ± 0.58^{a}	6.10 ± 0.34^{a}
-	5.0	0.00 ± 0.00^{c}	13.00 ± 0.55^{b}	5.70 ± 0.21^{a}
-	7.5	0.00 ± 0.00^{c}	10.20 ± 0.73^{c}	5.00 ± 0.23^{a}
-	10.0	0.00 ± 0.00^{c}	8.20 ± 0.58^{c}	3.76 ± 0.18^{b}

Values represent means ± standard error of 10 replicates per treatment in 3 repeated experiments. Means followed by the same letter not significantly different according to Tukey's test at 0.05 probability level.

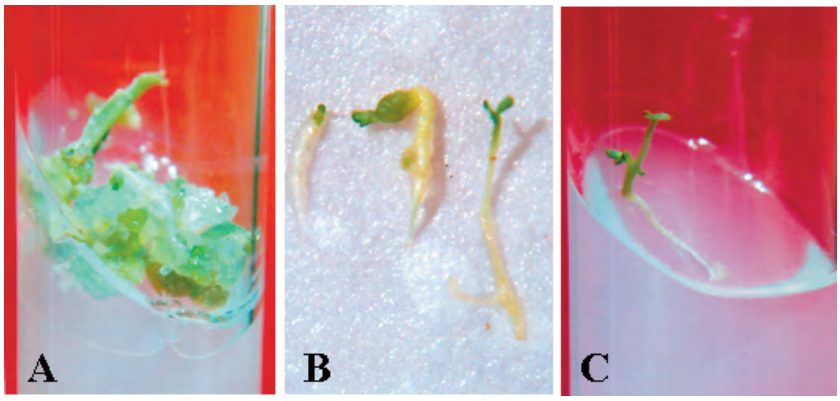

Figure 2. A) Callus showing germinating embryoid on MS + BA (5 μM).
B) Various stages of embryoid development isolated from callus on MS + BA (5 μM).
C) Mature, well developed embryoid on half-strength MS medium.

(90%) with 56 ± 3.6 shoots/culture through petiole derived callus. In *Astragalus cicer* L., TDZ was found to induce high frequency shoot induction along with low frequency somatic embryo formation (Basalma et al., 2008). Thus, TDZ was observed to be more effective than BA for shoot bud induction as compared to somatic embryogenesis; this is in accordance with the observations made by Thomas and Philip (2005) in *T. indica* wherein TDZ was found superior to BA and kinetin in terms of percentage of shoot organogenesis.

The slightly loose yellowish green calli produced on TDZ (2.5 μM) when transferred to BA (5 μM) supplemented MS medium led to the induction of somatic embryogenesis within 2 weeks of subculturing. Compared to previous reports the low frequency of shoot organogenesis in the present study on TDZ (2.5 μM) induced calli could be explained by the presence of determined embryogenic cells. TDZ might be suppressing the morphogenesis in these cells; thus when this callus was transferred to BA (5

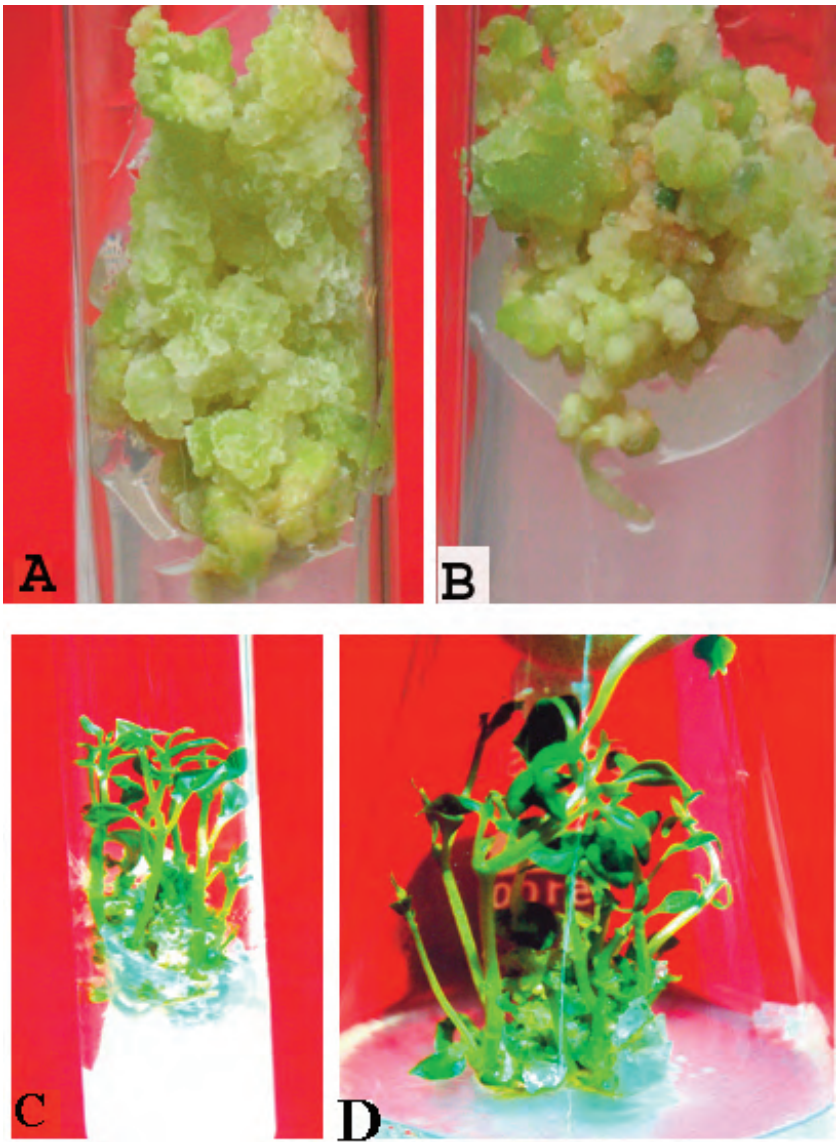

Figure 3. A) Yellowish green slightly loose callus induced on MS + TDZ (2.5 µM) after 4 weeks.
B) Six-week-old slightly loose callus on MS + TDZ (2.5 µM) showing embryogenesis and shoot bud induction.
C) High frequency multiple shoot induction on MS + BA (5 µM).
D) High shoot multiplication on proliferation medium containing MS + BA (2.5 µM).

µM) the effect of TDZ was nullified and the cells reverted back to embryogenesis. An inhibitory and deleterious effect of TDZ has been well documented by Huettman and Preece (1993).

Effect of repeated subculturing on:

Multiplication and proliferation of shoot buds

For further multiplication of shoot buds the middle nodular callus was subcultured on medium with BA (5 µM). This resulted in high frequency shoot bud induction along with an increase in number of shoots (23.4 ± 0.93 shoots/culture) up to 3 subculture passages (Table 2) (Figure 3C). Later these calli were transferred to proliferation medium in flasks containing a slightly lower concentration of BA (2.5 µM) (Figure 3D).

Table 2. Effect on shoot regeneration of repeated subculturing on BA (5 μM) of MS + BA (5 μM) and MS + TDZ (2.5 μM) derived callus.

Subculture Passage	BA (5 μM) derived callus		TDZ (2.5 μM) derived callus	
	Mean no. of shoots/culture	Mean shoot length (cm)	Mean no. of shoots/culture	Mean shoot length (cm)
I	15.00 ± 1.14[b]	5.30 ± 0.21[a]	19.40 ± 0.51[c]	6.20 ± 0.34[a]
II	21.00 ± 1.14[a]	5.60 ± 0.18[a]	21.40 ± 1.12[bc]	6.20 ± 0.14[a]
III	23.40 ± 0.93[a]	5.60 ± 0.17[a]	26.80 ± 0.97[a]	5.96 ± 0.16[a]
IV	20.00 ± 1.14[a]	5.20 ± 0.20[a]	25.20 ± 0.37[a]	5.80 ± 0.14[a]
V	19.60 ± 1.03[a]	5.10 ± 0.10[a]	24.40 ± 0.40[ab]	5.40 ± 0.06[a]

Values represent means ± standard error of 10 replicates per treatment in 3 repeated experiments. Means followed by the same letter not significantly different according to Tukey's test at 0.05 probability level.

Multiplication of embryoids

High frequency embryoid multiplication 9.8 ± 0.66 embryoid/culture was achieved on subsequent subculturing of the peripheral portion of calli to 5 μM of BA containing MS medium (Table 3). This is in contrast to the findings of Chandrasekhar et al. (2006) in *T. indica* where TDZ + 2,4 D combination medium was found effective in the induction of somatic embryos in leaf explants. However, Sudha and Seeni (2006) achieved somatic embryogenesis in *Rauvolfia micrantha* Hook.f. on MS medium supplied with NAA or IBA in combination with BA. Somatic embryogenesis through root explants was achieved on MS media augmented with BA (5.36 μM) in *T. indica* with a very low frequency (Chaudhuri et al., 2004). However, in *Podophyllum peltatum* L. embryogenesis was achieved on NAA and 2,4-D containing MS medium (Kim et al., 2007). The number of somatic embryoids also increased on repeated subculturing of TDZ derived calli onto BA (5 μM) augmented MS basal medium until 3 subculture passages (Table 3).

Conversion of somatic embryos into complete plantlets

Somatic embryos at various stages of development were isolated from the callus after every subculture and transferred to plant growth regulator free half-strength MS medium for further maturation where they developed into complete plantlets. Root and shoot initiation took place simultaneously in these bipolar structures.

Table 3. Effect on embryoid multiplication of repeated subculturing on BA (5 μM) of MS + BA (5 μM) and MS + TDZ (2.5 μM) derived callus.

Subculture Passage	BA (5 μM) derived callus		TDZ (2.5 μM) derived callus	
	% Response	Mean no. of embryoids/culture	% Response	Mean no. of embryoids/culture
I	70	6.60 ± 0.24[b]	70	5.20 ± 0.37[c]
II	80	8.00 ± 0.45[ab]	70	8.40 ± 0.40[b]
III	90	9.80 ± 0.66[a]	90	10.20 ± 0.37[a]
IV	80	8.60 ± 0.51[ab]	80	8.80 ± 0.37[ab]
V	80	8.40 ± 0.51[ab]	70	8.40 ± 0.40[b]

Values represent means ± standard error of 10 replicates per treatment in 3 repeated experiments. Means followed by the same letter not significantly different according to Tukey's test at 0.05 probability level.

Ten-day-old germinating somatic embryoids when transferred to germination medium consisting of half strength MS medium were converted to plantlets within 3 weeks of transfer. The same was reported earlier by Jayanti and Mandal (2001), where somatic embryoids transferred to basal medium developed into complete plantlets. This is probably due to restoration of the endogenous hormone balance necessary for normal plantlet development (Chaudhuri et al., 2004). About 90% of somatic embryos underwent conversion to plantlets, with a mean survival frequency of 50%.

Effect of various auxins on rooting

Microshoots (3-4 cm) excised from BA (5 µM) were individually transferred to root induction medium containing various concentrations of IBA and NAA (Table 4). Roots were induced within 7 days of incubation on IBA (0.5 µM) while in NAA rooting was delayed. Different morphological variations were observed in roots induced at various concentrations of auxins.

The best results were obtained on IBA supplemented half-strength MS medium compared to NAA. IBA was reported to be superior to NAA and IAA for root induction in *T. indica* by Thomas and Philip (2005). Success of IBA for efficient root induction is also reported in *Swainsona formosa* (G.Don) Joy Thomps. (Jusaitis, 1997), *Cunila galioides* Benth. (Fracaro & Echeverrigaray, 2001), *Melia azedarach* L. (Shahzad & Siddiqui, 2001), *Mentha arvensis* L. (Shahzad et al., 2002), and *Acacia sinuata* (Lour.) Merr. (Shahzad et al., 2006).

In general green and pigmented direct root regeneration was induced on IBA (Figure 4A) while whitish roots were obtained on NAA supplemented half-strength MS medium (Figure 4B). Thickness and number of lateral roots increased with increases in concentrations of IBA, while very thick deformed roots were obtained at all concentrations of NAA (Table 4). Similar observations were recorded by Chaudhuri et al. (2004) in *T. indica*, where NAA in particular and higher concentrations of IBA resulted in stunted and hypertrophied roots. IBA is known to suppress root elongation with increase in concentration (Koroch et al., 2002).

The rooting of shoots through basal callusing is not recommended because in most cases proper acclimatisation of the plantlet could not be achieved. Thus, direct root induction in microshoots is described as the most advantageous for successful establishment of the plant in natural conditions.

Acclimatisation

Acclimatisation is the most crucial stage for success of any in vitro regeneration protocol. Successful establishment of in vitro regenerated plantlets in field conditions requires great care. Plantlets with fully expanded leaves and well developed roots after removing the nutrient medium through gentle washing were transferred in thermocol cups containing soilrite for the purpose of hardening (Figure 4C, D). These were kept in culture room conditions for 20 days. Initially the plantlets were covered with glass bottles to avoid infection; later the plants were exposed by gradually increasing the time of exposure.

Table 4. Effect of IBA and NAA on root induction in in vitro raised microshoots of *T. indica*.

Basal Medium	IBA (µM)	NAA (µM)	% Response	Mean no. of roots/culture	Mean root length	Remark (cm)	Acclimatisation
½ MS	0.1	-	90	0.80 ± 0.20^{c}	7.16 ± 0.41^{b}	pigmented roots	70%
,,	0.5	-	100	5.80 ± 0.37^{a}	13.20 ± 0.80^{a}	,,	90%
,,	2.5	-	90	3.80 ± 0.37^{b}	9.20 ± 0.37^{b}	,,	80%
,,	5.0	-	80	3.60 ± 0.24^{b}	9.20 ± 0.37^{b}	,,	80%
,,	-	0.1	80	1.40 ± 0.24^{b}	10.00 ± 0.45^{a}	white roots	60%
,,	-	0.5	70	1.00 ± 0.32^{b}	5.50 ± 0.37^{b}	,,	70%
,,	-	2.5	70	1.60 ± 0.24^{b}	4.90 ± 0.19^{b}	,,	80%
,,	-	5.0	80	5.80 ± 0.37^{a}	2.14 ± 0.20^{c}	,,	80%

Values represent means ± standard error of 10 replicates per treatment in 3 repeated experiments. Means followed by the same letter not significantly different according to Tukey's test at 0.05 probability level.

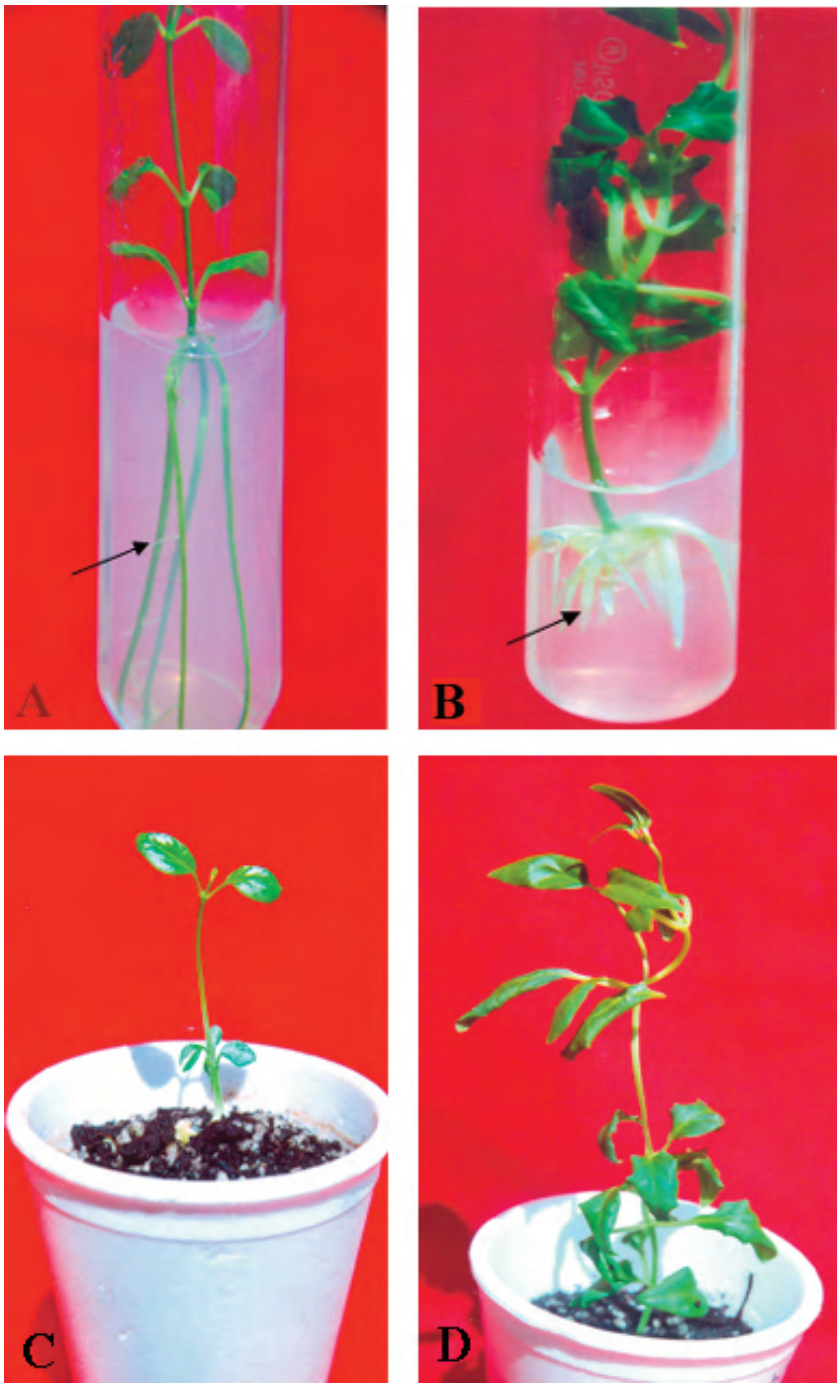

Figure 4. A) Green pigmented roots induced on MS + IBA (0.5 µM).
B) Whitish roots induced in plantlet on MS + NAA (5 µM).
C,D) Six-week-old acclimatised plantlets of *Tylophora indica*.

Hardened plants were then transferred to pots and kept in field conditions in a greenhouse. Initially these plants were kept in the shade for a few days before being planted out. A 90% survival rate was recorded. One-year-old plants are currently growing normally under field conditions.

Conclusion

An efficient and cost effective protocol has been established for the conservation of this endangered medicinal climber via organogenesis and somatic embryo induction. Further encapsulation of somatic embryos will generate an efficient system for germplasm transfer of *T. indica*.

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