BIOLOGIA PLANTARUM 49 (2): 257-260, 2005

BRIEF COMMUNICATION

An efficient *in vitro* method for mass propagation of *Tylophora indica*

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Abstract

A protocol of high frequency shoot organogenesis and plant establishment from stem derived callus has been developed for *Tylophora indica* (Burm. f.) Merrill. - an endangered medicinal plant. Callus was developed on Murashige and Skoog (MS) medium supplemented with 10 µM 2,4,5-trichlorophenoxy acetic acid (2,4,5-T). Multiple shoot induction was achieved from the surface of the callus after transferring onto shoot induction medium. The highest rate (80 %) of shoot multiplication was achieved on MS medium containing 5.0 µM kinetin. The developed shoots rooted best on half-strength MS medium supplemented with 0.5 µM indole-3-butyric acid (IBA). The *in vitro* raised plantlets with well developed shoot and roots were acclimatized successfully and grown in greenhouse.

Additional key words: callus culture, growth regulators, medicinal plant, shoot multiplication, 2,4,5-trichlorophenoxy acetic acid.

Tylophora indica (*Asclepiadaceae*) is a perennial climbing plant native to the plains, forest and hills of southern and eastern India. The pharmacological importance of this plant is mainly due to the presence of alkaloid tylophorine and tylophorenine. Besides, root contains a potential anti-tumor alkaloid tylophorinidine (Mulchandani *et al.* 1971).

The over-exploitation of *Tylophora indica* from the nature and inadequate efforts for its cultivation resulted in marked decline in the population of this species. Therefore, it is necessary to develop methods for *in vitro* propagation and conservation of this plant. Only micropropagation through axillary bud sprouting (Sharma and Chandel 1992) has been reported on this plant. It has been shown that shoot organogenesis *via* an intermediate callus phase can be used as an effective method for multiplication of other medicinal plants (Sarasan *et al.* 1994, Ahroni *et al.* 1997, Castillo and Jordan 1997, Sharma and Wakhlu 2001, Sin and Teng 2002). The present communication is the first report on an efficient *in vitro* propagation and multiplication of *Tylophora indica via* stem callus culture.

The young shoots of *Tylophora indica* collected from plants grown at the Botanical Garden, Aligarh Muslim University, were washed under running tap water for at least 30 min, followed by soaking in 5 % (v/v) *Teepol* for 5 min. After a thorough washing in sterile distilled water, the source tissues were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 3 min. Following repeated washes with sterile distilled water, the stem segments were cut into appropriate size (0.5 - 1 cm) and transferred onto culture medium.

The nutrient medium consisted Murashige and Skoog (1962; MS) salts and vitamins and 3 % (m/v) sucrose was used in all experiments. The pH of medium was adjusted to 5.8 prior to the addition of 0.8 % (m/v) agar and autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. The cultures were incubated at 25 ± 2 °C under 16-h photoperiod (irradiance of 50 µmol m^{-2} s^{-1}) and relative humidity of 60 %.

For callus induction from stem segments, four concentrations (0.5 - 10 µM) of each 2,4-dichloro-phenoxy acetic acid (2,4-D) and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) were tested. MS medium lacking

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Received 10 June 2003, *accepted* 18 October 2004.

Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxy acetic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; 2,4,5-T - 2,4,5-trichlorophenoxy acetic acid.

Acknowledgements: We express our sincere thanks to Dr. H.C. Chaturvedi, Emeritus Scientist (CSIR) and Former Head, Plant Tissue Culture Laboratory, The National Botanical Research Institute, Lucknow, India for valuable suggestions and critical evaluation.

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growth regulators served as control. For shoot induction and proliferation, the light yellow friable callus was transferred onto MS medium supplemented with either kinetin or 6-benzyladenine (BA) at four concentrations 0.5, 2.5, 5.0 and 10 µM and 5.0 µM kinetin (6-furfuryl-aminopurine) supplemented with 0.5 - 5.0 µM α-naphthalene acetic acid (NAA). The regenerated shoots measuring 4 - 5 cm in length were excised from callus clump and cultured on MS basal medium supplemented with 0.5 - 5.0 µM indole-3-acetic acid (IAA) or 0.5 - 5.0 µM indole-3-butyric acid (IBA) and on auxin-free full and half strength MS medium.

The *in vitro* regenerated plantlets with well developed shoots and roots were washed with distilled water and transferred to pots containing sterile vermiculite under diffuse light (16-h photoperiod). Potted plants were covered with transparent polythene membrane to ensure high humidity, and watered every three days with half strength MS-salt solution free of sucrose for 2 weeks. After one month, plants were transferred to garden soil in glasshouse and watered with tap water.

All the experiments were repeated three times with 20 replicates. The data were analyzed using one-way analysis of variance (*ANOVA*) and means were compared using the Duncan's multiple range test at 0.05 level of significance.

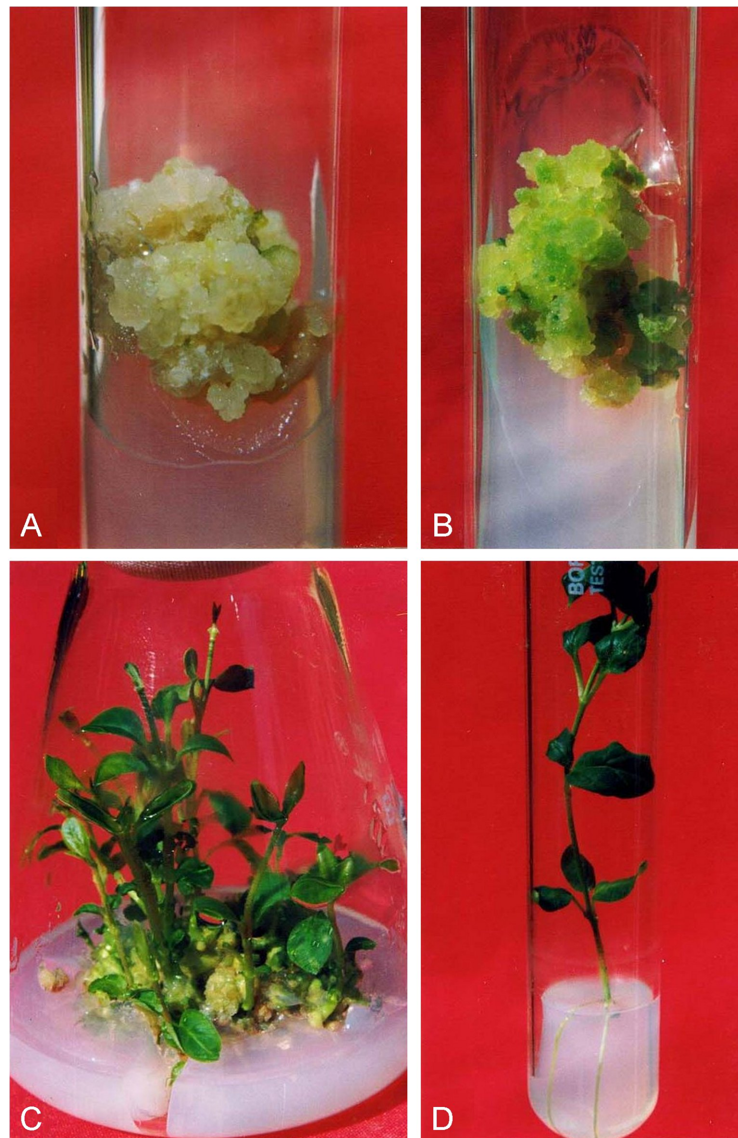

Fig. 1. Induction of callus, shoot regeneration and *in vitro* rooting of *T. indica*. *A* - callus induction from stem segments on MS + 2,4,5-T (10 µM); *B* - differentiation of shoot buds on MS + kinetin (5.0 µM); *C* - shoot proliferation and multiplication on MS + kinetin (5.0 µM); *D* - root developed in half-strength MS medium with IBA (0.5 µM).

Stem explants cultured on MS medium supplemented with 2,4-D or 2,4,5-T exhibited callusing after 10 d of inoculation but morphology and growth of callus was affected by the concentration of 2,4-D and 2,4,5-T. About 70 % cultures showed callusing at lower concentration of 2,4-D. On increasing its concentration upto 10 µM, about 95 % cultures formed callus. The stem explants produced highly proliferating light yellow friable callus on the medium containing 2,4,5-T. On increasing the concentration of 2,4,5-T from 0.5 to 10 µM, a gradual increase in percentage of cultures forming callus was observed. The highest frequency (100 %) of organogenic callus was obtained on the medium containing 10 µM 2,4,5-T (Fig. 1*A*).

When the light yellow friable callus was subcultured on MS medium containing different concentration of kinetin or BA alone, it became greenish, nodular and more organized. Adventitious shoot buds were observed from the surface of callus within four weeks (Fig. 1*B*). Formation of leaves and shoot elongation occurred within 6 weeks (Fig. 1*C*). The callus culture inoculated on MS medium responded differently to two different cytokinin (kinetin/BA) concentration (Table 1). The highest regeneration frequency (80 %) and maximum number (45.0 ± 1.10) of shoots were achieved with 5.0 µM kinetin. Increasing the concentration of kinetin upto 10 µM, a decrease in regeneration ability was noticed.

On MS medium supplemented with 0.5 to 10 µM BA, the highest regeneration frequency (70 %) and average shoot number (38.0 ± 0.92) was recorded at 5.0 µM BA.

Table 1. Effect of plant growth regulators on shoot regeneration from stem derived callus of *T. indica* in MS medium after 6 weeks of culture. Means ± SE, *n* = 60. Means followed by the same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level.

Kinetin [µM]	BA [µM]	NAA [µM]	Regeneration [%]	Shoot number [callus^{-1}]
0.5			15.0 ± 1.5^{f}	24.2 ± 0.85^{cf}
2.5			62.0 ± 3.0^{c}	32.0 ± 1.08^{c}
5.0			80.0 ± 3.5^{a}	45.0 ± 1.10^{a}
7.5			70.5 ± 2.7^{b}	25.5 ± 0.64^{dc}
10.0			40.0 ± 2.0^{d}	19.7 ± 0.85^{g}
	0.5		10.7 ± 2.2	21.7 ± 1.11^{fg}
	2.5		45.0 ± 4.0^{d}	27.5 ± 1.32^{d}
	5.0		70.2 ± 2.3^{b}	38.0 ± 1.92^{b}
.	7.5		50.0 ± 2.0^{c}	19.2 ± 0.86^{g}
	10.0		40.0 ± 3.4^{d}	16.0 ± 1.50^{h}
5.0		0.5	50.0 ± 2.4^{c}	12.0 ± 1.30^{i}
5.0		1.0	40.5 ± 3.0^{d}	20.0 ± 2.10^{g}
5.0		2.5	20.3 ± 1.5^{e}	14.5 ± 1.04^{hi}
5.0		5.0	20.0 ± 1.6^{e}	8.5 ± 1.29^{j}

Shoots regenerated on BA supplemented medium showed slow growth and poor internodal elongation in comparison to the shoot regenerated from kinetin supplemented medium which revealed better elongation. The effect of NAA in combination with the optimal concentration of kinetin was also tested. The addition of NAA reduced the rate of shoot regeneration (Table 1).

The success of *in vitro* regeneration relies on the rooting percentage and survival of the plantlets in field conditions. Half-strength growth regulator free MS medium was found superior to full-strength MS medium in promoting rooting. The incidence of root formation on auxin-free medium may be due to the availability of endogenous auxin in *in vitro* shootlets (Minocha 1987). The presence of auxin (IAA or IBA) at lower concentration in half-strength MS medium facilitated better rhizogenesis. Half-strength MS medium fortified with IBA was found superior to IAA with respect to the induction of roots (Table 2). The maximum frequency of root formation (90 %) was achieved in half-strength MS medium containing 0.5 µM IBA (Fig. 1*D*). The effectiveness of IBA in rooting has been reported for medicinal plants like *Hemidesmus indicus* (Sreekumar *et al.* 2000), *Aloe polyphylla* (Abrie and Van Staden 2001), *Rauvolfia tetraphylla* (Faisal and Anis 2002) and *Sesbania drummondii* (Cheepala *et al*. 2004).

Table 2. Effect of MS strength and IAA or IBA concentrations on root induction from in *vitro* raised shoots of *Tylophora indica* after four weeks of culture. Means ± SE, *n* = 60. Means followed by the same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level.

Medium	IAA [µM]	IBA [µM]	Rooting [%]	Root number [shoot^{-1}]
MS			50 ± 2.1^{d}	1.80 ± 0.41^{cd}
½MS			75 ± 3.2^{b}	2.70 ± 0.15^{bc}
½MS	0.5		75 ± 4.0^{b}	3.67 ± 0.30^{ab}
½MS	2.5		50 ± 2.6^{d}	1.96 ± 0.26^{cd}
½MS	5.0		15 ± 3.4^{f}	1.40 ± 0.13^{d}
½MS		0.5	90 ± 4.1^{a}	4.30 ± 0.47^{a}
½MS		2.5	70 ± 3.7^{c}	2.08 ± 0.23^{cd}
½MS		5.0	30 ± 1.7^{e}	1.76 ± 0.14^{cd}

Hardening and acclimation of all the *in vitro* plantlets was completely successful. Compared to mother plants, the *in vitro* raised plants did not show any phenotypic variation.

In conclusion, the present communication presents an efficient protocol for large scale mass propagation and conservation of *Tylophora indica.* This *in vitro* regeneration procedure provides the step towards the development of transformation system of this endangered species.

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