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RESEARCH ARTICLE

Leaf as a Potential New Source of Competent Cells for High Frequency Regeneration through Indirect Organogenesis in *Tylophora Indica* (Burm.f.) MERRILL (Asclepiadaceae)

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ABSTRACT

The purpose of this study was to develop a new micropropagation system for *Tylophora indica*, an important medicinal plant in India, using leaf explants as starting material. Leaf explants cultured on Murashige & Skoog's (MS) medium supplemented with 2mg/L NAA and 1mg/L BAP, compact greenish white callus resulted from leaf explants. From leaf explants on MS + 2mg/L 2,4-D + 0.75 mg/L BAP and MS + 2.5mg/L 2,4,5-T + 0.5 mg/L BAP, friable callus was obtained. The compact callus also yielded friable callus when transferred to B_5 medium supplemented with 0.75 mg/L 2,4-D and 0.2 mg/L NAA. These calli were non morphogenetic. Morphogenetic compact callus derived from leaf explants differentiated shoots on MS + 1.0mg/L 2ip + 4mg/L KIN. Good rooting response was observed when regenerated shoots were inoculated in ½ MS with 2.0% sucrose. Such plantlets ere successfully transferred to soil after hardening, with a high rate of survival. The plants were comparable to natural population in growth in vigour.

KEYWORDS: Organogenesis, *Tylophora indica*, Micropropagation, Asclepiadaceae, Callus.

INTRODUCTION:

Tylophora indica (Burm.f.) Merrill (Asclepiadaceae) is a perennial climber native to the plain and hilly forests of eastern southern India up an altitude of 900 m. This indigenous medicinal plant has been traditionally used by tribes in certain regions of India for the treatment of various ailments (Anonymous, 1976). The plant contains several phenanthroindolizidine alkaloids (Gallert, 1982), and pharmacological investigations have confirmed the anti-asthmatic effects of its leaf extracts (Shivpuri *et al.*, 1972). The major alkaloid present-tylophorin has been reported to have immunosuppressive, anti-inflammatory (Gopalakrihsnan *et al*., 1980). The leaf and stem extracts as well as the minor alkaloid present tylophoridine are known to possess anti-leukemia properties (Galler, 1982). Several pharmaceutical companies (Acorn Chemicals, India; Sabinsa, USA) are presently marketing the extract of *Tylophora indica* as anti-asthmatic herbal drugs (Chaudhuri *et al*., 2004). Due to its significant medicinal properties Tylophora indica has been over exploited and a lack of organized cultivation has led to a rapid decline in the wild population of this species (Jayanthi and Manda, 2001). Micropropagation of *T. indica* by axillary shoot induction and adventitious shoot production (Sharma and Chandel, 1992) and callus mediated somatic embryogenesis from leaf (Jayanthi and Mandal 2001) and stem (Rao and Narayanaswamy, 1972) and root (Chaudhuri *et al*., 2004) explants have been reported. However, to date the indirect organogenesis that is the induction of multiple shoots from the callus derived from the leaf has not been reported in T. indica. The objective of the investigation reported here was to develop a rapid and reproducible in vitro regeneration system from leaf explants of T. indica for mass propagation of selected elite clones. *In vitro* techniques can enable the mass propagation of this herb from minimum of plant material facilitating large quantities of biomass required for extraction of the active/anti-asthmatic principle that can be made available throughout the year. Here we have reported the establishment of a regeneration system from leaf tissue of T. Indica involving the pathway indirect organogenesis.

MATERIAL AND METHODS:

Young healthy shoots of T. indica were collected during the month between June-July from 3 year old plants growing at the herbal garden maintained by the Department of Biotechnology, Sathyabama University, Chennai, India. Fresh leaves were first washed in running tap water to remove debris and soil from the surface and soaked in 5% (v/v) liquid detergent (Tween 20, Himedia, India) for 10 minutes, then washed under running tap water. Those leaves then surface sterilized with 0.05% (w/v) $HgCl_2$ for 12-15 min and thoroughly rinsed four or five times with sterile distilled water. The explants (10 mm) were excised and placed on solid medium. The basal medium used in all experiment was MS's (Murashige and Skoog, 1962) medium with 3% (w/v) sucrose and 0.8 % (W/v) agar (Himedia, India). Dependingon the experiment, the basal medium was supplemented with various plant growth regulators as required (in the tables). The pH of the media was adjusted to 5.8 ± 0.02 and dispensed in 20 l aliquots into culture tubes (15 x 2.5 cm, Borosil, India) and

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capped with plugs of non-adsorbent cotton prior to autoclaving at 121º C for 15 minutes. Cultures in all experiments were incubated in the culture room maintained under 16/8 hrs (light/dark) photoperiod at 25 ± 1 º C under cool white fluorescent tubes (Philips, India) at an intensity of 50µ mol $m^{-2}s^{-1}$ and 75-80 % relative humidity. Each experiment was repeated thrice with 10 replicates per treatment. Various combinations of plant growth regulators and readymade media were tried for shoot differentiation and rooting of the plant regenerated from callus. The rooted plants were washed and shifted to greenhouse condition in small poly cups (covered with a transparent plastic bag with holes to maintain humidity) containing soil and sand mixture (1:1). Subsequently they were transferred to the garden and after one month they were planted in the field. Standard error is given to indicate the variation among the means of three experiments based on 10 replicates for each treatment. The data regarding shoots and roots were collected after 35 days and 25 days, respectively, after inoculation and were analyzed by ANOVA with a confidence limit of 0.05.

RESULTS AND DISCUSSION:

Through consistent observation the results have been reported here. Callus originating from herbaceous explants regenerate much better than those from woody plants (Pierik, 1987). Leaf explants when cultured on MS medium supplemented with α-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) or N^6-benzyl adenine (BA) produced no callus. At 1.5-2.5 mg/L BAP, moderate amount of callus was obtained. In MS medium augmented with 1.5 mg/L and 2.0 mg/L BA, leaf explants with midrib initially showed callus formation but after 8 weeks it gradually turned black and died. High frequency callus formation was obtained from leaf explants on MS medium supplemented with NAA-BA and NAA-KIN. The callus was compact, nodular and pale green. High frequency callus formation was obtained from leaf explants on MS medium supplemented with 2,4-D and BA and also with 2,4,5-T and BA. The leaf callus when transferred MS medium with 2 mg/L 2,4-D and 0.75 mg/L BA or 2.5 mg/L 2,4,5-T and 0.5 mg/L BA produced friable callus. The compact callus, when sub cultured on to B_5 medium with 0.75 mg/L 2,4-D and 0.2 mg/L NAA generated highest frequency of friable callus (Table 2, Fig. 1-C). The nodular callus when transferred to ½ MS gave rooting percent 30% only. The explants of *T. indica* inoculated on MS medium containing 2,4-D and 2,4,5-T (0.2-3.0 mg/L) showed differentiation of multiple shoots, the numbers increased with increasing concentration of 2,4-D (highest being 2 & 2.5 mg/L) without intervening callus formation in *Curculigo orchioides* (Prajapati *et al*., 2003). The morphogenetic compact callus from leaf explants cultured on MS medium supplemented with MS + 1.0 mg/L 2ip + 4 mg/L KIN showed shoot differentiation after 6-7 weeks. Earlier a limited number of shoots (average 2-3) regenerated from leaf callus on MS basal medium (Table 3, Fig. 1-E & G). The combination of 0.2 mg/L NAA and 0.2-2.5 mg/L BA tried for shoot differentiation showed varied response (Table 3). Prolonged culture of *T. indica* differentiated roots with ½ MS basal medium with 2% sucrose proved best for rooting. ½ MS with 1 % sucrose produced lesser roots per culture though the percentage response was good (86%). These results indicate that lower nutrient levels of MS salts are suitable for rooting when compared with full MS (Table 4). NAA and IBA tried with ½ MS (2.0% sucrose) induced rooting at lower concentrations. With IBA less profuse rooting with thick, few primary roots were obtained. In ½ MS (2.0 % sucrose) with various concentrations of NAA varied response resulted, the lower concentrations promoted best rooting response. At higher concentrations (1.5-3.0 mg/L) the callus multiplied from the base. The regenerated plantlets did not show any detectable phenotypic variation. For medicinal species it is highly essential to retain the quality and quantity of desired secondary metabolites (Natesh, 2001). The ultimate success of in vitro propagation lies in the successful establishment of plants in the soil (Saxena & Dhavan, 1999). Direct transfer to sunlight caused charring of leaves and wilting of the plants. The regenerated plantlets were transferred to poly cups containing sterilized soil for acclimatization at 25 ± 1º C for 20 days during which elongation and growth of leaves was observed (Fig.1-K). Later, these plantlets were transferred to green house, kept for 30 days and then transferred, pots containing garden soil and sand in 1:1 ratio. The pots containing the plantlets were finally transferred to the garden plot after two weeks. The in vitro grow plants exhibited survival rate of 85.75 % and 70.50% in green house and field condition, respectively. The high survival rate of in vitro plants of Tylophora indica indicates that this procedure could be easily adopted for large-scale cultivation.

CONCLUSION:

A simple and reliable protocol for in vitro propagation of *Tylophora indica*, an important medicinal plant through indirect organogenesis i.e., the production of multiple shoots from leaf explants through callus induction has been achieved. The protocol ensures rapid multiplication of this important herb throughout the year, thus reducing the risk of its extinction.

Sr. No.	Different concentration and combination of PGRS used	Percentage of Response	Nature of callus
1	MS + 0.5 mg/l NAA + 1.0 mg/l BA	68.2 ± 0.8	C, G, Md
2	MS + 1.0 mg/l NAA + 1.0 mg/l BA	74.7 ± 0.2	C, G, Lg
3	MS + 1.5 mg/l NAA + 1.0 mg/l BA	84.2 ± 0.7	C, G, Lg
4	MS + 2.0 mg/l NAA + 1.0 mg/l BA	87.5 ± 0.4	C, G, Md
5	MS + 2.5 mg/l NAA + 1.0 mg/l BA	80.3 ± 0.5	C, G, Md
6	MS + 3.0 mg/l NAA + 1.0 mg/l BA	78.6 ± 0.3	C, G, Lg
7	MS + 1.0 mg/l NAA + 2.0 mg/l BA	66.4 ± 0.6	C, P, Lg
8	MS + 1.5 mg/l NAA + 2.0 mg/l BA	72.4 ± 0.5	C, G, Lg
9	MS + 2.0 mg/l NAA + 2.0 mg/l BA	68.6 ± 0.3	C, P, Md
10	MS + 2.5 mg/l NAA + 2.0 mg/l BA	72.8 ± 0.9	C, G, Lg

Table No. 1- Effect of plant growth regulators as MS supplements on callus induction from the leaf explants of *Tylophora indica* after 6 weeks.

Values are mean ± SD
Lg – Large, Md – Moderate, C- Compact, G- Greenish White, P – Pale green.

Sr. No.	Different concentration and combination of PGRS used	Percentage of Response	Nature of callus
1	MS + 1.0 mg/l 2,4-D + 0.75 mg/l BA	66.2 ± 0.4	F, Md
2	MS + 2.0 mg/l 2,4-D + 0.75 mg/l BA	74.6 ± 0.2	F, Lg
3	MS + 0.5 mg/l 2,4,5-T + 0.5 mg/l BA	64.2 ± 0.6	F, Md
4	MS + 1.5 mg/l 2,4,5-T+ 0.5 mg/l BA	67.5 ± 0.4	F, Md
5	MS + 2.5 mg/l 2,4,5-T +0.5 mg/l BA	73.8 ± 0.5	F, Lg
6	B_5 + 0.25 mg/l 2,4-D + 0.2 mg/l NAA	70.4 ± 0.6	F, Lg
7	B_5 + 0.50 mg/l 2,4-D + 0.2 mg/l NAA	76.4 ± 0.4	F, Lg
8	B_5 + 0.75 mg/l 2,4-D + 0.2 mg/l NAA	88.6 ± 0.6	F, Lg
9	B_5 + 0.25 mg/l 2,4-D + 0.4 mg/l NAA	72.8 ± 0.4	F, Lg
10	B_5 + 0.50 mg/l 2,4-D + 0.4 mg/l NAA	76.8 ± 0.2	F, Lg

Table No. 2- Effect of plant growth regulators as MS supplements on callus subculture of *Tylophora indica* after 6 weeks.

Values are mean ± SD
Lg – Large, Md – Moderate, F- Friable.

Sr. No.	Different concentration and combination of PGRS used	Percentage of Response	Mean No. of Shoots	Mean length of shoots (cm)
1	MS	$76.8 \pm 1.2_b$	$1.72 \pm 0.7_y$	8.9 ± 0.5
2	½ Ms	$79.4 \pm 1.4_a$	$1.96 \pm 0.3_y$	8.3 ± 0.6
3	MS + 0.5 mg/l BA	$64.6 \pm 0.4_c$	$0.97 \pm 0.1_z$	7.4 ± 0.6
4	MS + 1.0 mg/l BA	$71.2 \pm 0.2_c$	$1.04 \pm 0.6_z$	7.9 ± 0.2
5	MS + 0.2 mg/l 2ip + 1mg/l KIN	$68.2 \pm 0.4_d$	$1.01 \pm 0.1_z$	8.9 ± 0.6
6	MS + 0.2 mg/l 2ip + 3mg/l KIN	$72.4 \pm 0.6_e$	$1.06 \pm 0.4_z$	9.4 ± 0.2
7	MS + 0.2 mg/l NAA + 0.5 mg/l BAP	$57.6 \pm 0.4_f$	$3.01 \pm 0.5_x$	8.3 ± 0.6
8	MS + 0.2 mg/l NAA + 1.0 mg/l BAP	$65.2 \pm 0.2_g$	$3.05 \pm 0.3_x$	9.5 ± 0.8
9	MS + 0.2 mg/l NAA + 1.5 mg/l BAP	$64.9 \pm 0.2_g$	$2.65 \pm 0.4_x$	8.9 ± 0.4
10	MS+ 0.2 mg/l NAA + 2.0 mg/l BAP	$49.8 \pm 0.6_h$	2.78 ± 0.6	8.3 ± 0.2

Table No. 3- Effect of plant growth regulators on shoot formation in 8 week old leaf callus of *Tylophora indica* after 6 weeks.

Mean values within the column followed by the same letters are not significantly different at P<0.05 (F-LSD Test)

Sr. No.	Media used	Percentage of Response	Mean No. of roots	Mean length of roots (cm)
1	MS	65.6 ± 0.4	$8.8 \pm 1.2_c$	19.3 ± 0.4
2	MS*	67.4 ± 0.2	$9.1 \pm 0.9_c$	21.5 ± 0.6
3	MS^s	70.4 ± 0.6	$8.6 \pm 1.1_c$	18.7 ± 0.2
4	½ MS	80.2 ± 0.2	$18.6 \pm 1.4_a$	21.2 ± 0.4
5	½ MS*	83.0 ± 0.4	$19.3 \pm 0.5_c$	23.2 ± 0.2
6	½ MS^s	86.8 ± 0.2	$15.8 \pm 1.9_b$	19.8 ± 0.2
7	¼ MS	72.5 ± 0.2	$17.5 \pm 1.0_a$	24.7 ± 0.6
8	¼ MS*	75.8 ± 0.4	$15.7 \pm 1.3_b$	20.4 ± 0.4
9	¼ MS^s	77.3 ± 0.6	$16.5 \pm 1.8_b$	21.5 ± 0.6

Table No. 4- Influence of nutrient levels of MS medium on in vitro rooting of *Tylophora indica*

Mean values within the column followed by the same letters are not significantly different at P<0.05 (F-LSD Test)

*Indicates 2.0 % sucrose

^{s} Indicates 1.0% sucrose

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