Research Article

REGULATION OF ORGANOGENESIS USING LEAF, INTERNODE AND PETIOLE EXPLANTS IN *TYLOPHORA INDICA* (BURM. F.) MERR.

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ABSTRACT

The research study regarding the shoot formation from leaf, internode and petiole explants via organogenesis, subsequent developments and rooting of the *in vitro* proliferated shoots of *Tylophora indica* (Burm. f.) Merr. and transplantation of regenerated plants under *in vivo* condition has been done. Organogenic calli were obtained from leaves of *Tylophora indica* on MS medium supplemented with BAP (2.0 mg/l) and IBA (0.5 mg/l). Out of different explants tried viz leaf, internode and petiole, leaf gave the optimum organogenic calli with 95% response on MS medium supplemented with BAP (2.0 mg/l) and IBA (0.5 mg/l) in combination. The elongation of developed shoots (12.00±1.50 cm) was obtained on MS medium along with TDZ (0.1 mg/l). Maximal rooting responses (85%) were obtained by the subculture of regenerated shoots on half strength of MS medium supplemented with IBA (0.5 mg/l) after 15 days of incubation. Complete plantlets were hardened, acclimatized and successfully transferred to natural environmental conditions, where they showed 75% survival rate.

Keywords: *In vitro* regeneration, Organogenesis, Callus, *Tylophora indica.*

INTRODUCTION

Tylophora indica (Burm. f.) Merr. a perennial, branching climber of the family Asclepiadaceae, is medicinally important plant, particularly for the treatment of asthma and bronchitis[1, 2]. The roots have a sweetish taste turning acrid, aromatic odor and a brittle fracture and also possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic properties due to which it is being used for the treatment of whooping cough, dysentery, diarrhoea and in rheumatic gouty pains[3, 4]. The powdered leaves, stem, and roots contain several alkaloids including tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$) and anticancerous tylophorinidine ($C_{22}H_{22}O_4N$)[5, 6, 7].

These potent alkaloids endowed this plant with a variety of medicinal value to cure various ailments. Owing to these pharmaceutical and medicinal properties, this plant has been overexploited and at the same time, its natural propagation through seeds is successful with minimum chances of germination, because seed viability is poor[7]. Keeping in view all these above growth constrains and its immense value to medicinal and pharma fields, its multiplication at a large scale is needed, which can only be fulfilled by the tissue culture techniques.

Furthermore, plant tissue and cell culture are also important tool, which allow extensive manipulation of the biosynthesis of secondary compounds and yield a higher productivity as compared to that of intact plants[8].

Although, the reports on regeneration protocol through tissue culture has been available previously[9-13]. However, all the available protocols are useful but when tried were not repeatable and efficient. At the same time, here we produced a reliable, repeatable and highly efficient regeneration protocol, which would be useful for pharmaceutical and tissue culture companies, and is repeatable also.

MATERIALS AND METHODS

Explant Sterlization

One year old *T. indica* plant was obtained from World Arboretum, Jaipur and planted in pots in the Department of Botany, University of Rajasthan, Jaipur for regular procurement of explants for experimentation. For experimental work, leaf, internode and petiole explants procured and were kept under running tap water for about 15-20 minutes and then kept in a solution of liquid detergent (Teepol 1% (v/v) for 2-4 minutes so as to remove the adhered soil particles. They were then rinsed with sterile double distilled water at least thrice to remove the traces of detergent. Surface sterilized explants were again treated with 0.1% mercuric chloride solution (w/v; $HgCl_2$) for 2-3 minutes prior to their inoculation. These surface sterilized explants were then aseptically inoculated on sterile MS medium[14] having a wide range and combinations of PGR. The medium was supplemented with various growth regulators (Auxin: IBA, NAA, 2,4-D and Cytokinin: BAP) and pH of the medium was adjusted to 5.8±0.02 before autoclaving at 121°C for 15 minutes on 1.06Kg/cm^{-3} pressures.

Media preparation and culture condition

All the cultures were maintained in an air conditioned culture room at a temperature of 26±2°C. The source of illumination consisted of 4 feet wide fluorescent tubes (Philips, 40W). The intensity of illumination was 2500±500

lux, 16/8 hour photoperiod and 55±5% humidity was maintained.

Various concentrations of BAP (0.5-3.5 mg/l) for callus initiation and auxin such as IBA (0.025-3 mg/l), NAA (0.5-5.0 mg/l) and 2,4-D (0.5-5.0 mg/l) for further differentiation of calli were used for the organogenesis process though leaf, internode and petiole explants.

Callus development and morphogenesis

The experiments were conducted to initiate and produce stock callus for further morphogenic growth through leaf, internode and petiole explants. These explants were separately inoculated on MS medium fortified with different concentrations of BAP (0.5-3.5 mg/l) to initiate callus. Further, after initiation of callus, they were subcultured gradually and separately on optimum concentration of BAP along with a array of various auxins like IBA (0.025-3 mg/l), NAA (0.5-5.0 mg/l) and 2,4-D (0.5-5.0 mg/l) for the production of stock calli. Furthermore, the clump of calli were tested for the morphogenic purposes.

Shoot elongation and plant recovery

The organogenic callus having adventitious shoot buds derived from leaf explants were then transferred to elongation medium. For this MS medium along with various concentrations of TDZ (0.05-0.5 mg/l) was tried.

***In vitro* rooting and transfer of plantlets to natural environment (acclimatization)**

The *in vitro* elongated shoots were then transferred on ½ MS medium supplemented with various concentrations of different auxin: IBA, IAA and NAA (0.05-0.5 mg/l) for *in vitro* root induction. The plantlets were then gently picked up from culture vessel without damaging the delicate root system and then rinsed with distilled water to remove adhering agar. These plantlets were then transferred to polycups containing, vermicompost and autoclaved soil (1:3). Plants were covered with inverted glass beakers to maintain high humidity for hardening.

RESULTS AND DISCUSSION

In the present research work, the medium was supplemented with various concentrations of different growth hormones for the induction of callus from various explants like petiole, internode and leaf taken from field grown mature plants. Growth hormone BAP alone could initiate callusing from petiole, internode and leaf explants but the growth of callus was slow. For further experimentation, leaf explants were used. The caulogenic effect of BAP through leaf explants observed in the present study, is in consonance with other reports[15-18].

Petiole explants also showed callusing on Murashige and Skoog basal medium supplemented with BAP (2.0 mg/l) and 2,4-dichlorophenoxyacetic acid (2.0 mg/l) in combination (Fig. C). At the same time, Haensch[19] obtained high-frequency shoot bud formation and plant establishment of *Madame Layal* (*Pelargonium* x *domesticum*) from petiole derived callus was also in favoure of the results obtained during the study. In contrary to this, Faisal and Anis[20] reported that 2,4-D, 2,4,6-T and TDZ in combination was optimum for orgenogenic callus establishment in *Tylophora indica*.

Moreover, callus initiation was induced from internode explants on MS medium augmented with BAP (2.0 mg/l) and NAA (3.0 mg/l) within 3 weeks of inoculation (Fig. B). A number of publications available in support of the present results for callus induction from internode explants in *Amsoina orientalis* Decne.[21] and *Ipomoea obscura*[22]. In contrast to the above result, Singh *et al.* [23] obtained optimum callus on BAP and IAA in *Rauvolfia serpentina* L.

For further studies, leaf (3-5mm^2) segments were cultured on MS medium augmented with BAP (2.0 mg/l) and IBA (0.5 mg/l) proved to be optimum for stock callus production after 3 weeks of inoculation (Fig. A). Similar results were also noticed in *Melia azedarach* L.[24]. In contrast, *Lin et al.* [25] reported that Kn is better than BAP to induce callusing from leaf explants in case of *Ocimum santum*.

Initially, callusing started at the cut ends and later on whole of the leaf segment covered with callus and within three weeks, the entire explant turned into a mass of soft, green and friable callus. Leaf explants also showed callus induction on MS medium supplemented with BAP (2.0 mg/l) but the growth of callus was slow & took nearly 45 days for completely turning into a mass of callus.

Plant regeneration via indirect shoot organogenesis has been achieved from the culture of leaf explants. Leaf segments were more responsive as compared to internode and petiole explants for callus induction and its further proliferation. However, internode and petiole explants were tried for complete regeneration of shoots through indirect method but they only restricted to only shoot regeneration. Further development of these microshoots was not attained.

High frequency callus mediated shoot regeneration can be utilised for the induction of somaclonal variation for the improvement of this valuable medicinal plant. Callus mediated shoot morphogenesis has been well documented in several medicinal plants such as *Tylophora indica*[26,20], *Saussurea obvallata*[27], *Euphorbia nivulia*[28] and *Cassia angustifolia*[29].

During the study, leaf derived callus was subcultured for further division and proliferation of shoot buds on MS medium supplemented with BAP (2.0 mg/l) and IBA (0.5 mg/l) (Fig. D). The optimized medium having BAP (2.0 mg/l) and IBA (0.5 mg/l) showed emergence of 15.22±0.21 microshoots often 2 weeks of subculturing. After that, the *in vitro* regenereted micro shoot buds were carefully and aspectically transfered to the elongation medium having TDZ. Of the different concentrations tried, TDZ at 0.1 mg/l gave maximum shoot elongation (12.00±1.50 cm) (Table 1) (Fig. F).

Table 1: Effect of plant growth regulators for callus induction, their further proliferation into multiple shoots through leaf, internode and petiole explants

Plant growth regulators (mg/l)				Leaf derived calli		Internode derived calli		Petiole derived calli	
BAP	NAA	2,4-D	IBA	% response	Number of shoots/explants *Mean±S.E.$t_{0.05}$	% response	Number of shoots/explants *Mean±S.E.$t_{0.05}$	% response	Number of shoots/explants *Mean±S.E.$t_{0.05}$
0.5				20	2.25±0.10	17	1.10±0.15	12	0.80±0.10
1.0				35	2.88±0.50	24	2.00±0.10	20	0.90±0.22
1.5				40	3.15±0.22	36	2.20±0.21	26	1.05±0.10
2.0				**60**	6.11±1.50	**54**	3.50±0.40	**36**	2.40±0.24
2.5				46	5.50±0.40	45	2.88±0.11	25	2.06±0.16
3.0				30	4.90±0.10	31	2.55±0.21	22	1.20±0.55
3.5				23	3.10±0.20	20	2.40±0.12	10	0.50±0.10
2.0	0.5			19	2.20±0.10	30	2.90±1.05	14	1.25±0.12
2.0	1.0			22	2.45±0.15	44	3.54±0.90	28	1.80±0.15
2.0	2.0			35	2.75±0.25	65	4.80±1.20	38	2.90±0.25
2.0	3.0			37	3.55±1.50	**85**	**6.54±1.60**	36	2.95±0.45
2.0	4.0			28	3.40±0.40	62	4.75±0.95	25	2.45±0.20
2.0	5.0			16	2.90±0.10	48	4.25±0.20	19	1.88±0.40
2.0		0.5		9	0.90±1.05	8	0.14±0.50	23	2.30±0.86
2.0		1.0		12	1.15±0.22	12	0.86±0.12	35	2.90±0.60
2.0		2.0		20	1.91±0.54	23	0.96±0.69	**45**	**3.96±0.45**
2.0		3.0		31	3.15±0.42	26	1.53±0.58	32	2.95±0.50
2.0		4.0		24	2.56±0.56	34	2.01±0.14	28	2.65±0.58
2.0		5.0		21	2.14±0.15	29	1.90±0.58	19	2.05±0.22
2.0			0.025	80	11.14±0.90	25	2.24±0.12	15	1.60±0.30
2.0			0.5	**95**	**15.22±0.21**	45	3.36±0.45	35	2.80±0.60
2.0			1.0	75	9.28±1.00	35	3.00±0.50	30	2.62±0.54
2.0			2.0	68	8.75±0.80	28	2.75±0.25	22	1.95±0.76
2.0			3.0	55	6.45±0.25	20	2.44±0.22	18	1.54±0.36

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Table 2: Influence of half strength of MS salts along with various auxins on *in vitro* rooting response

Plant growth regulators (mg/l)		% response of rooting	No. of roots per cuttings *Mean± S.E. $t_{0.05}$	Days taken for the emergence of roots
IBA	IAA			
0.05		10%	1.10±0.41	15-16
0.1		28%	2.25±0.24	12
0.2		35%	3.14±0.56	11-12
0.3		45%	4.45±0.58	11
0.4		72%	6.65±0.85	10
0.5		**85%**	**7.75±0.25**	8
	0.05	5%	1.05±0.10	14-16
	0.1	15%	1.08±0.51	13
	0.2	25%	2.10±0.65	12-13
	0.3	45%	4.45±0.52	11
	0.4	35%	3.35±0.54	12
	0.5	22%	2.36±0.26	13

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

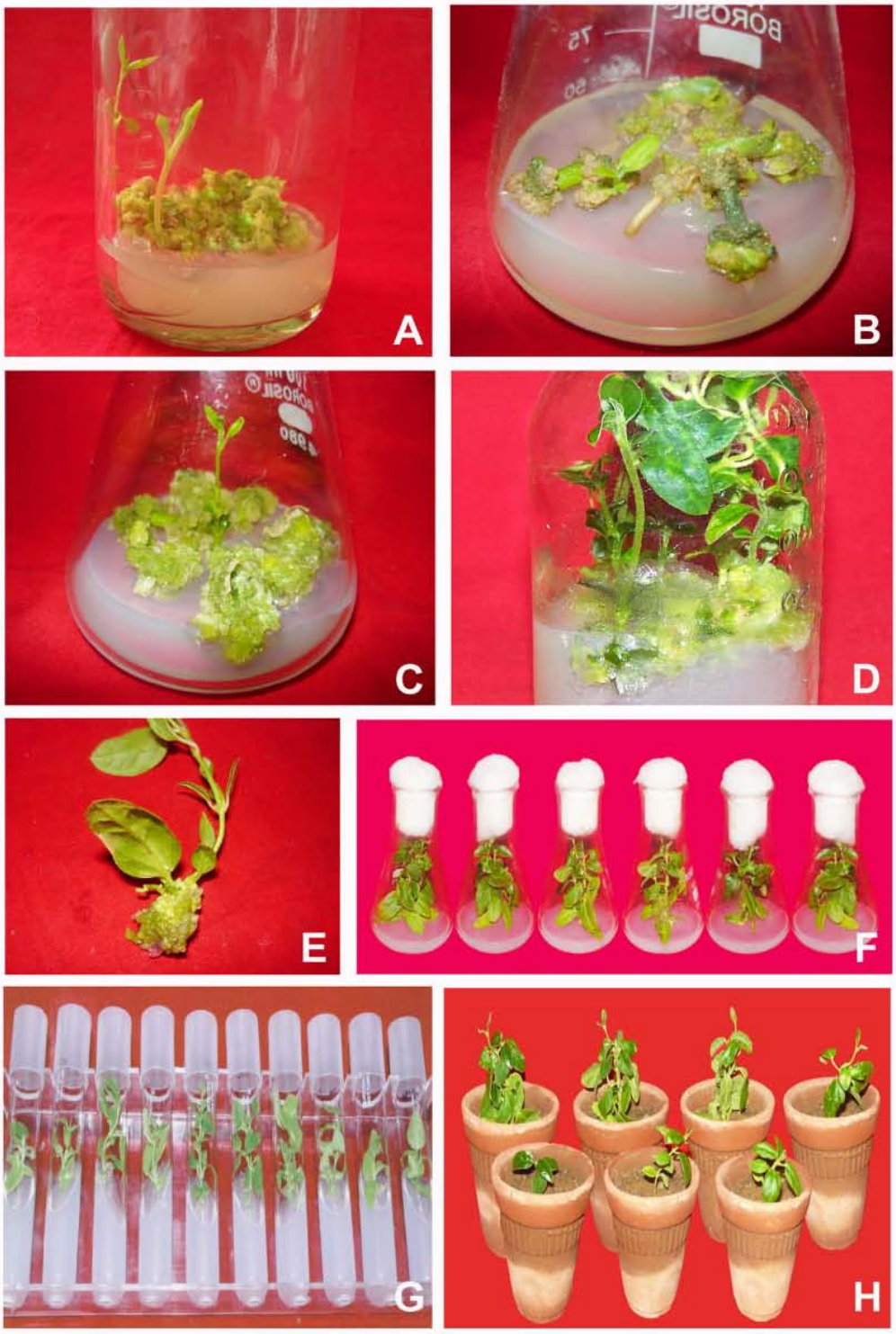

Fig. A- Shoot differentiation from leaf on MS medium supplemented with BAP (2.0 mg/l) + IBA (0.5 mg/l).

Fig. B- Shoot differentiation from internode on MS medium supplemented with BAP (2.0 mg/l) + NAA (3.0 mg/l).

Fig. C- Shoot differentiation from petiole on MS medium supplemented with BAP (2.0 mg/l) + 2,4-D (2.0 mg/l).

Fig. D- Multiple shoot regeneration from the indirect organogenesis of leaf explants after 3 weeks of subculturing.

Fig. E- Expose view of differentiation.

Fig. F- Shoot elongation on MS medium supplemented with TDZ (0.1 mg/l).

Fig. G- Root initiation on ½ MS medium supplemented with IBA (0.5 mg/l).

Fig. H- Hardened plantlet of *T. indica* growing in garden soil.

Similar results were also reported by Banerjee *et al.* [30] in *Cineraria maritima* Linn. In contrast to the above results, Vijyalakshmi and Giri,[31] observed that BAP and IAA showed better shoot regeneration and elongation in *Arachis stenosperma* and *Archis villora*, respectively.

Root induction is an important and crucial step in the formation of complete plantlets, usually controlled by the treatment with growth regulators, where an auxin plays key role in the formation of adventitious roots[32]. *In vitro* elongated shoots were separated and transferred to half strength of MS medium supplemented with various concentrations of IBA (Fig. G). Out of all the concentrations tried, IBA at 0.5 induced rooting without formation of callus at the cut end. At the same time, the role of IAA for *in vitro* shoot induction has been presented in Table 2. No rooting response was observed on NAA supplemented medium. Similarly, Ryugo and Breen[33] proposed that the principle role of IBA is to favour the conjugation between endogenous IAA and amino acid, which leads to the synthesis of the specific proteins necessary for the formation of root initiation. However, at the same time, IBA in other studies also proved to be optimum in other plant species like Phyllanthus amerus Shum. and Thonn[16], Murraya koenegii L. Spreng. [17], *Mentha arvensis*[34] and *Hoya wightii* spp. *Palniensis*[35]. However, in oppugnance to the above results, Dhabhai *et al.* [36] in *Acacia nilotica* and Escutia *et al.* [37] in *Tigridia pavouia* (L.F.) DC found NAA to be the best for rooting.

These complete plantlets having 9-10 nodes and 20-21 leaves taken out from the culture vials and washed their roots with distilled water delicately and transferred to earthen pots followed by hardening and acclimatization as mentioned in “materials and methods” (Fig. H). After hardening, these plantlets transplanted in natural environmental conditions, where they showed 75% of survival rate.

In conclusion, the present work which is an attempt to investigate the dedifferentiation & redifferentiation responses of cells of various organs of *Tylophora indica* to varied & diverse chemical milieu corroborates the concept of totipotency as proposed by Haberlandt[38] & supported the contention of Mehra and Mehra[39].

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