In Vitro Propagation of *Tylophora indica*-Influence of Explanting Season, Growth Regulator Synergy, Culture Passage and Planting Substrate

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Abstract: An efficient protocol for rapid clonal propagation of an endangered medicinal plant, *Tylophora indica* (Burm. *f.*) Merrill through *in vitro* culture is described. High frequency bud break (85%) and multiple shoot formation were induced from nodal segments explanted between September through November and cultured on MS medium supplemented with 2.0mg/l BAP. Although callus- free multiple shoot formation was a function of cytokinin activity alone, faster bud break coupled with enhanced frequency of shoot development (95%) and internode elongation were dependent on the synergistic effect of GA_3(0.2mg/l). By repeated sub culturing of nodal segments harvested from the newly formed axenic shoots, prolific shoot cultures, free of proximal callusing, showing a high frequency multiplication rate were established within three months. The percentage shoot multiplication as well as the number of shoots per node attained the highest values (100%, 7 shoots/node) during the first two culture passages; beyond this there was a gradual decline in shoot bud differentiation. Rooting of the excised shoots from secondary or subsequent cultures was best induced on ½ strength MS medium containing 0.5 mg/l IBA. Vermi-compost was the most suitable planting substrate for hardening and its use ensured high frequency survival (96%) of regenerated plantlets prior to outdoor transfer. Regenerated plants get established in pots containing garden soil followed by their transfer to natural soil under full sun. The *in vitro* regenerated plants were uniform and identical in growth characteristics and morphology to the donor plants. [Journal of American Science 2010;6(12):385-392]. (ISSN: 1545-1003).

Keywords:*Tylophora indica,* medicinal plant, micropropagation, culture media, explants, growth regulators

1. Introduction

Biotechnology has provided several unconventional techniques for crop improvement and its use is revolutionizing traditional plant breeding methods. Plant propagation *via* organogenesis is one of the techniques used in plant tissue culture to obtain large number of plants, irrespective of season and with conservation of space and time. Organogenesis has been reported in many species and factors influencing it has also been studied intensively (Koroch *et al*., 2002; Thao *et al*., 2003; Yemets *et al*., 2003).

Tylophora indica (Burm *f.*) Merrill (Asclepiadaceae) is a threatened medicinal climber shrub native to the plain and hill forests of eastern and southern India up to an altitude of 900 m. The plant has been traditionally exploited by tribes in certain regions of India for the treatment of various ailments (Anonymous, *1976*). The plant contains several phenanthroindolizidine alkaloids (Gellert, *1982*) and pharmacological investigations have confirmed the anti-asthmatic effects of its leaf extracts (Shivpuri *et al*., *1972*). The major alkaloid present—tylophorine—has been reported to have immunosuppressive, anti-inflammatory (Gopalakrishnan *et a*l., *1980*) and anti-tumor (Donaldson *et al*., *1968*) properties. The powdered leaves, stems and roots also contain other minor alkaloids (Rao and Wilson, 1971) including tylophorinine, cryptopleurine, antofine and ficuseptine C which are pharmacologically active and anticancer tylophorinidine has also been isolated from the roots of three-year old plant (Mulchandani *et al*., 1971). Thus the plant is in great demand for the production of traditional and modern medicines.

Owing to large scale and uncontrolled exploitation of this natural resource in order to meet its ever-increasing demand in the pharmaceutical companies, wild stock of this plant species has been markedly depleted over past few years. Unfortunately, efforts for its replenishment by conventional cultivation have been handicapped, because it is not amenable to vegetative propagation through cuttings, and propagation through seeds would result in variation. Therefore, the application of a reliable, *in vitro* clonal propagation system would provide an alternative method of propagation to meet the pharmaceutical needs and for effective conservation of this precious plant species. It is advantageous for conservation of germplasm and multiplication of best genotypes with high alkaloid contents.

Although micro propagation of *T. indica* by axillary shoot induction and adventitious shoot production (Sharma and Chandel, *1992;* Faisal *et al.*,

2007) and callus-mediated somatic embryogenesis from leaf (Jayanthi and Mandal, *2001;* Chandrasekhar *et al*., 2006) and inter nodal (Thomas, *2006*) explants have been previously reported but these studies were of preliminary nature and more studies and further refinements of techniques were absolutely necessary. Hence in the present investigation we evaluate various factors influencing *in vitro* axillary shoot proliferation and resulted in an efficient and reproducible procedure for rapid clonal multiplication of this pharmaceutically important plant species.

2. Material and Methods

2.1 Plant material and sterilization

During different month's healthy nodal explant (0.4-0.6 cm) were collected from plants of *T. indica* from 5-year-old plants grown in the medicinal plant garden of CCS HAU, Hisar (Haryana, India). The explants were initially soaked in 5% (v/v) liquid detergent (Teepol, Reckitt and Colman, India) for 10 min, then washed under running tap water for 30 min and rinsed in distilled water. The explants were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and thoroughly rinsed four or five times with sterile double distilled water.

2.2 Culture media and conditions

The basal culture medium used for the present study was MS (Murashige and Skoog, 1962) medium supplement with 100mg/l (w/v) myo-inositol and 3% (w/v) sucrose. To this medium was further added 0.25-5.0 mg/l BAP(6-Benzylaminopurine) or Kin (Kinetin) and 0.1-0.5 mg/l GA_3 (Gibberellic acid)) was tested either individually or in combination with BAP or KIN. The pH of the media was adjusted to 5.8±0.1 before gelling it with 0.8% (w/v) agar (Merck, India) and dispensed in 25-ml aliquots into 200 ml screw capped glass jars (Excel glasses Ltd. Allepey, India) or 150 ml Erlenmeyer flasks (Borosil, India) prior to autoclaving at 121°C for 20 min. The surface disinfected explants were implanted horizontally on the culture medium (5 explants per jar or flask). Cultures in all experiments were incubated in a growth chamber maintained at 24±1°C and 60-65% relative humidity under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 48 µmol m^{-2} s^{-1}.

2.3 Multiplication of shoots

Primary shoots formed *in vitro* were isolated and sectioned into one-node pieces after removing the leaves. These nodal segments, each containing the axillary bud, were cultured on MS medium fortified with 2.0 mg/l BAP+0.2mg/l GA_3 for further multiplication. Subsequent subcultures were in the same medium at a periodic interval of 4 weeks.

2.4 Rooting of shoots

For root induction, the shoots (ca. 4-5 cm high) with three to five leaves were harvested from secondary cultures and transferred to ½ strength MS medium containing 2% (w/v) sucrose and 0.8% (w/v) agar. The medium were further supplemented with 0.5-2.0 mg/l IAA (Indole-3-Acetic Acid), NAA (α-Napthaleneacetic Acid) or IBA (Indole Butyric Acid). Data were recorded on the percentage of rooting, the mean number of roots per shoot and the root length after four weeks of transfer onto the rooting medium.

2.5 Acclimatization and transfer of plantlets to soil

Plantlets with well developed roots were removed from the culture medium and after washing the roots gently under running tap water; the plantlets were transferred to plastic pots containing autoclaved garden soil or artificial soil or vermi-compost moistened with autoclaved tap water. Two different types of artificial soil were examined viz. vermiculite and soilrite mix. The potted plantlets were maintained inside the plant growth chamber set at 25±1°C, 80–85% relative humidity under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 50 µmol m^{-2} s^{-1}. After 30 days the plantlets were transplanted to earthenware pots containing garden soil and kept under shade in green house for another 2 weeks before transferring to outdoors under full sun.

3. Results

3.1 Shoot Proliferation

Bud breakage and development of shoots from nodal explants was a function of cytokinin activity. The morphogenic responses of nodal explants collected during September through November to various concentrations of cytokinins are shown in Table 1. There was no sign of bud break even after 30 days on MS basal medium without any growth regulator supplement. Of the two cytokinins tested BAP was more effective than Kin in inducing bud break as well as multiple shoot formation. There was a linear correlation between the increase in concentration of BAP up to the optimum level (2.0 mg/l) and percentage shoot development. The number of shoots per explant also increased with increments in BAP concentrations up to the optimum level (2.0 mg/l). MS containing 2.0 mg/l BAP induced bud break in ca. 84% of the nodal explants. The explants cultured on the medium showed their first response by an initial enlargement of the existing axillary bud following bud break within 10-12 days. From each developing bud a single shoot elongated within 12-15 days. These shoots attained a height averaging 4.20 cm in 30 days bearing 4-6 leaves (Table 1). All

regenerated shoots were free from callus formation at their proximal ends. The percentage bud break and induction declined with the increase in BAP concentration beyond the optimal level (2.0 mg/l, Table 1). Above 2.0 mg/l, BAP caused a suppression of sprouting. Multiple shoots were induced from each sprouted bud at higher concentrations of BAP (5.0 mg/l), but these failed to elongate (Table 1). On the other hand, when MS containing a singular supplement of GA_3 was used, the sprouting period was lengthened, but percentage bud break and shoots/explant were substantially reduced. A medium of only 14-16% of the axillary buds sprouted within 18-20 days of culture on MS with an optimal concentration of GA_3 (0.2 mg/l) alone. Only one or two shoots were formed which attained a height averaging 4.5 cm within 30 days of culture. Concentrations of GA_3
higher than 0.2 mg/l had no promotive influence on percentage shoot development and on shoot number per explant. However, there was a progressive increase in shoot length at increasing GA_3 concentrations. A combination of optimal concentration of BAP (2.0 mg/l) and GA_3 (0.2 mg/l) in the culture medium not only induced a faster bud break (within 7 days) but also enhanced the frequency of bud break (ca. 95%). In addition a BAP-GA_3 coupling had a synergistic effect on multiple shoot formation (Fig. 1A) as well as on internode elongation (6.5 cm high shoots after 30 days, Table 1). The synergistic effect was also noticed in a combination of Kin (2.0 mg/l) and GA_3 (0.3 mg/l), but with respect to percentage shoot development, shoot number and shoot length it was inferior to BAP- GA_3 coupling.

3.2 Influence of Explanting Season on Culture Establishment

The shoot proliferation was greatly influenced by the month of the year during which the explants had been collected. Highest frequency bud break (95%) coupled with maximum number of shoots formed (four to five shoots/explant) occurred with nodal explants excised between September through November representing autumn season in India. Other explanting periods were comparatively less suitable (Table 2), the period between December to February (winter season) being the least responsive.

3.3 Nodal Multiplication of Axenic Shoots

Nodal segments excised from the primary, *in vitro* raised shoots cultured on MS containing 2.0 mg/l BAP plus 0.2 mg/l GA_3 produced six to eight shoots per node within 4 wk. By repeated subculturing of nodal segments from the newly formed axenic shoots harvested from each culture passage at every 4 week interval, prolific shoot cultures free from basal callusing was established within three months. The percentage shoot multiplication as well as the number of shoots per node attained the highest values (100%, 7 shoots/node) during the first two culture passages, beyond which there was a gradual decline in shoot bud differentiation (Fig.2).

3.4 Rooting of regenerated shoots

The *in vitro* regenerated shoots were transferred to ½ MS medium with or without auxins. Root formation from the basal cut portion of the shoots was observed one week after transfer to the rooting medium and rooting frequency gradually increased over time & reached a maximum after four weeks of culture. The presence of an auxin (IAA, IBA or NAA) at a low concentration in ½ MS medium was found to be more effective for rooting (Fig.1B) and the best rooting was achieved in the medium fortified with 0.5 mg/l IBA; fairly good shoot numbers (4.55 ± 0.40) and root lengths per shoot (5.25 ± 0.08) were obtained (Table 3).

3.5 Evaluation of Planting Substrates for Acclimatization Prior to Outdoor Transfer

Plantlets with five to six leaves and well developed roots were successfully hardened off in the environmentally controlled growth chamber in the selected planting substrates for 5 wk and eventually established in natural soil. Of the four different types of planting substrates used, the highest survival rates for the micropropagated plants were achieved in vermi-compost (96%, Fig.1C), followed in decreasing order by that in soilrite mix (82%), vermiculite (68%) and garden soil (66%) (Table 4). All plants survived following transfer from the vermi-compost to natural soil (Fig. 1D). There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

Table 1. Morphogenic Response of Nodal Explants of *T. indica* to Different Concentrations of Cytokinins (KIN, BAP) and/or GA_3

Growth Regulator(mg/l)	Shoot Development (%)	Shoot Number/Explant	Shoot Length (cm)
0.0	-	-	-
KIN			
0.25	28.86 ± 1.76	1.80 ± 0.00	1.38 ± 0.07
0.5	43.52 ± 1.32	1.12 ± 0.12	1.82 ± 004
1.0	57.96 ± 1.45	1.56 ±0.13	1.98 ± 0.03

2.0	61.86 ± 1.74	1.66 ± 014	2.86 ± 0.06
5.0	41.55 ± 1.46	1.35 ± 0.06	0.55 ± 0.04
BAP			
0.25	46.86 ± 1.36	1.21 ± 1.76	1.86 ± 0.11
0.5	67.52 ± 1.22	1.85 ± 1.32	2.88 ± 0.16
1.0	76.96 ± 1.35	3.66 ± 1.45	2.20 ± 0.17
2.0	84.86 ± 1.74	3.98 ± 1.64	4.10 ± 0.09
5.0	41.55 ± 1.46	1.55 ± 1.40	0.55 ± 0.10
GA_3			
0.1	14.50 ± 1.70	1.12 ± 1.45	4.0 ± 0.16
0.2	16.00 ± 0.24	1.18 ± 0.74	4.5 ± 1.00
0.3	12.90 ± 1.38	1.12 ±0.40	5.00 ± 0.45
0.4	10.86 ± 1.94	1.10 ± 0.24	6.10 ±0.74
0.5	10.00 ± 1.13	1.00± 0.13	6.55 ± 0.46
KIN + GA_3			
2.0 + 0.2	68.56 ± 1.76	2.66 ± 0.76	4.46 ± 0.78
2.0 + 0.3	74.52 ± 1.32	2.90 ± 0.30	5.52 ±0.22
2.0 + 0.4	72.90 ± 1.47	2.95 ±0.12	5.96 ± 0.43
BAP + GA_3			
2.0 + 0.2	95.00 ± 1.70	4.86 ± 1.76	6.80 ± 0.70
2.0 + 0.3	80.52 ± 1.82	4.50 ± 1.32	6.10 ± 0.35
2.0 + 0.4	77.96 ± 1.65	4.00 ± 0.45	6.00 ± 0.40

Data presented as the mean value ± standard error after 30 days of culture from four independent experiments each with 20 replicates.

Table 2. Influence of Explanting Period on Culture Establishment of *T. indica* on MS supplemented with BAP (2.0 mg/l) and GA_3 (0.2 mg/l)

Months of Collection	% Bud Break	Shoot Number/Explant
December – February	58.48 ± 1.15	2.30 ± 0.11
March – May	67.55 ± 2.40	2.25 ± 0.08
June – August	79.20 ± 2.35	4.10 ± 0.19
September - November	95.74 ± 3.19	4.50 ± 0.20

Data presented as the mean value ± standard error after 30 days of culture from four independent experiments each with 20 replicates.

Table 3. Effect of Auxins on root formation of *in vitro* formed shoots of *T. indica* in half-strength MS medium.

Auxins (mg/l)			Rooting (%)	Mean number of roots/shoot	Mean root length (cm)
IAA[1]	IBA	NAA			
–	–	–	70	2.70 ± 0.19	2.50 ± 0.30
0.5	–	–	70	3.65 ± 0.30	3.00 ± 0.25
1.0	–	–	50	1.90± 0.25	2.75 ± 0.20
2.0	–	–	20	1.48 ± 0.15	2.30 ± 0.10
–	0.5	–	90	4.55 ± 0.40	5.25 ± 0.08
–	1.0	–	65	2.20 ± 0.35	4.80 ± 0.19
–	2.0	–	28	1.74 ± 0.19	3.10 ± 0.17
–	–	0.5	79	3.75 ± 0.60	2.49 ± 0.25
–	–	1.0	54	2.40 ± 0.21	2.00 ± 0.11
–	–	2.0	19	1.70 ± 0.25	1.98 ± 0.10

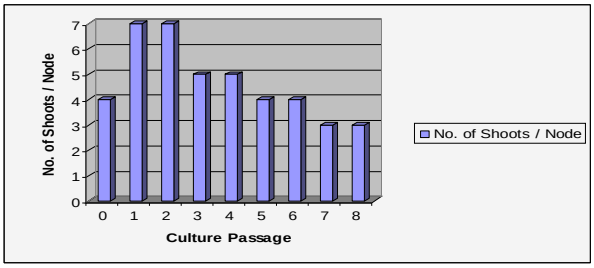
Data presented as the mean value ± standard error of roots formed after 30 days of culture from four independent experiments each with 20 replicates.

Table 4. Evaluation of Different Planting Substrates for Acclimatization of *In Vitro* Shoots

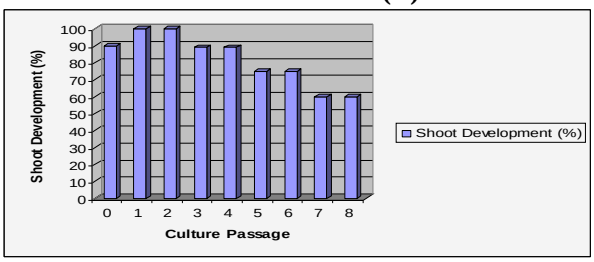
Planting Substrate	Number of Plants Transferred	Number of Plants Survived	Survival (%)	Shoot Height (cm)	Leaves/Plantlet	Root Length (cm)
Vermiculite	50	34	68	6.65 ± 1.19	6 ± 3	8.70 ± 0.12
Soilrite mix	50	41	82	12.65 ± 1.30	8 ± 1	8.60 ± 0.30
Vermi-compost	50	48	96	15.90± 1.15	10± 2	9.60± 0.25
Garden soil	50	33	64	8.48 ± 1.15	9 ± 1	6.40 ± 0.12

Data presented as the mean value ± standard error after 30 days of culture on planting substrates.

Figure 1. A-D.
A - Multiple Shoot formation from Nodal Explants of *T. indica* on MS + 2.0 mg/l BAP + 0.2 mg/l GA_3.
B – Rooted Shoot of *T. indica* on ½ MS + IBA (0.5mg/l)
C – A regenerated *T. indica* plant acclimatized on Vermi-compost.
D – *T. indica* plant established on natural soil.



(A)



(B)

Figure 2. A-B: Secondary shoot multiplication using nodal segments from primary -shoot cultures through successive culture passage (4 week) on MS + 2.0 mg/l BAP + 0.2 mg/l GA_3.

4. Discussions

Nodal segments containing axillary buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets. In nature, these buds remain dormant for a specific period depending on the growth pattern of the plant. However, using tissue culture techniques, the rate of shoot multiplication can be greatly enhanced by performing axillary bud culture in a nutrient medium containing suitable combinations and concentrations of growth regulators.

In the present study, the stimulatory effect of a singular supplement of BAP on bud break and multiple shoot formation in *T.indica* was similar to that reported earlier in other medicinal species including *Chlorophytum borivilianum* (Purohit *et al*., 1994) and *Ocimum spp.* (Pattnaik & Chand, 1996 and Sahoo *et al*., 1997). Our observations on the suppression of sprouting at higher BAP concentrations were in accordance with those of Pattnaik & Chand (1996) in *O. americanum* and *O. sanctum*. In another species of *Ocimum viz. O. basilicum*, although the nodal segments required the presence of BAP at 1.0 mg/l at the initial stage of bud break their further growth and proliferation demanded transfer to a medium containing BAP at a relatively low concentration (0.25 mg/l) (Sahoo *et al*. ,1997).

Singular supplement of GA_3 at an optimal concentration (0.2 mg/l) had promotive influence on shoot development but in *Plumbago indica* (Nitsch & Nitsch, 1967) and *Duboisia myoporoides* (Kukreja & Mathur, 1985), GA_3 has been shown to suppress shoot bud differentiation. Thus the role of GA_3 in shoot induction and development in medicinal plant species remains controversial.

The synergistic effect of BA in combination with an auxin has been demonstrated in many medicinal plants from the Asclepiadaceae family, such as *Gymnema sylvestre* (Reddy *et al*., *1998*), *Holostemma annulare* (Sudha *et al*., *1998*), *Hemidesmus indicus* (Sreekumar *et al*., *2000*), *Holostemma ada-kodien* (Martin ,*2002*), *Leptadenia reticulata* (Arya *et al., 2003*), and *Ceropegia candelabrum* (Beena *et al., 2003*). In accordance with these reports, the present work studied the effect of low concentration of cytokinin (BAP and Kin) in combination with GA_3 on shoot induction efficiency and BAP- GA_3 coupling had shown a noticeable synergistic influence on multiple shoot formation. The promotive effect of GA_3 in combination with BAP on shoot bud induction was also reported earlier in other perennial medicinal herbs including *O. americanum* and *O. sanctum* (Pattnaik & Chand, 1996 and Sahoo *et al*., 1997) and *Tridax procumbens* (Sahoo & Chand, 1998).

Fluctuations in environmental factors in different seasons had a definite effect on shoot bud differentiation from explanted nodal segments in *T. indica* as similarly shown in other medicinal herbs including *Ocimum* species (Pattnaik & Chand 1996).

The multiplication rate of the shoot cultures derived from the nodal explants of the primary shoots was dependent on the number of the subsequent culture passages. The gradual decline in the frequency of shoot development and the number of the shoots per node was also reported earlier for *Tridax procumbens* (Sahoo & Chand, 1998), is indicative of a gradual loss of the morphogenic potential with advance in culture.

To optimize the rooting response of plantlets raised *in vitro*, different auxins(IAA,IBA and NAA) were tested at various concentrations. IBA was found to induce a strong rooting response. The success of IBA in promoting efficient root induction has been reported earlier in other species by Sreekumar *et a*l., *2000* ; Fracaro and Echeverrigaray, *2001;* Martin, *2002*; Beena *et al*,. 2003; Faisal *et al*., 2006 and also in *Tylophora indica* by Faisal *et al*., 2007.

The period of transition after transfer from lab to land is the most critical step in the tissue culture techniques as during the process of *in vitro* regeneration in the lab, plants are subjected to heterotrophic mode of nutrition and there is lack of adaptation or exposure to the outside environment. So, during the period of hardening care is taken over the physical (temperature, light intensities, relative humidity, air current, atmospheric CO_2) and other factors (mineral nutrition, pH etc.) employed. Another

important factor during acclimatization is the type of potting material used. In the present study, Vermi-compost was found to be most suitable planting substrate for hardening and its use ensured high frequency survival (96%) of regenerated plantlets prior to outdoor transfer.

In conclusion, the present investigation has resulted in a protocol, which could be used for ex situ conservation and true to type mass propagation of this shrub of immense pharmaceutical relevance.

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