Journal of Experimental Sciences Vol. 1, Issue 11, Pages 04-06 [2010]
www.jexpsciences.com

Regular Article

In vitro Regeneration and Phytochemical Screening of *Tylophora indica*, an Endangered Medicinal Herb

S. Rathinavel and T. Sellathurai*

Plant Tissue Culture Lab, Centre for Research in Botany, Saraswathi Narayanan College (Autonomous), Madurai - 625 022, Tamil Nadu, India

ABSTRACT: *In vitro* regeneration of *Tylophora indica* from mature leaf explants placed on MS with low concentration (1μM) of Kin and BA alone and in combination with Kin and BA induced multiple shoots and roots. Leaf explants on MS with BA and 2, 4-D or NAA along with Ads had poor organogenic responses. The responses of leaf explants in MS + Kin (1μM) implicate the feasibility for multiplication of this species. Comparative phytochemical analysis revealed the presence of alkaloids, steroids and carbohydrates both in adult leaf and *in vitro* derived callus. It evidences the potential utility of tissue cultures for production of bioactive compounds. It may be concluded that further standardization of hormonal combinations could helpful for large scale propagation and extraction of drugs for pharmaceutical application.

Key words: *Tylophora indica*, Leaf, Growth regulators, Callus, Alkaloids

Abbreviations: BA – N^6- benzyladenine; Kin-kinetin; IAA- indole-3-acetic acid; MS- Murashige and Skoog's; NAA- α - naphthalene acetic acid; Ads – Adenine sulphate

Introduction

Tylophora indica (Burm.f) Merr. belonging to family Asclepidaceae, is an endangered important indigenous medicinal plant found in restricted localities in India. It is a laticiferous climber with long fleshy, knotty root. Traditionally, this plant finds use in the treatment of a number of diseases and is commonly used for curing asthma, bronchitis, whooping cough, dysentery, diarrhoea, hydrophobia, wounds, ulcer, dyspepsia, flatulence, haemorrhoids, gout, and vitiated conditions of vata. It is exploited only from wild population for extraction purposes due to non-availability of sufficient quality plant materials and lack of commercial plantations. Its large scale utility in pharmaceutical industries led to declining of this species from natural habitat. The conventional methods of propagation were not amenable for multiplication and conservation due to its sensitivity to varying agro-climatic conditions, low rate of seed set and seasonal dormancy. Moreover it is difficult to collect the seeds as they are dispersed by wind on attaining maturity in this system. Therefore, suitable *in vitro* propagation methods must be evolved to conserve this valuable medicinal species. Direct and indirect somatic embryogenesis and multiplication of shoots from explants like leaf (Bera and Roy, 1993; Manjula *et.al.,* 2000; Chandrasekhar *et.al.,*2006), shoot and stem segments(Faisal and Anis,2005) and petiole (Faisal *et.al.,*2006) have been reported using MS medium supplemented with various concentrations and combinations of growth regulators. But, phytochemical screening was neither made in the regenerated plants nor in the developed callus. In the present study, regeneration potential of mature leaf segments was assessed for clonal propagation as well as for commercial pharmaceutical application.

Materials and Methods

In vitro regeneration

Healthy leaves were collected from branching climber of *T.indica*, which was obtained from the forests of Western Ghats and maintained in the botanical garden at research Centre of Botany, Saraswathi Narayanan College, Madurai, India. The leaves were repeatedly washed with running tap water to remove adhering particles of living and non-living. This was followed with rinsing in 5% (v/v) liquid detergent solution (Teepol) for 16-20 min at room temperature. After thorough rinsing in sterile distilled water, they were washed in 70% (v/v) ethanol for 30 s and finally surface - sterilized in 0.1% (w/v) $HgCl_2$ solution for 7 min. The explants were then rinsed in sterile double distilled water three to four times. The leaves were cut into 0.5 cm^2 segments and cultured on Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose, and 0.8% (w/v) agar (Himedia). The basal medium was supplemented with different concentrations and combinations of cytokinins (benzyladenine and kinetin) and auxins (2,4-Dichlorophenoxy acetic acid and α-naphthalene acetic acid). The pH of the medium was adjusted to 5.8 after addition of the growth regulators and autoclaved at 1.06 kg cm^{-2} pressure and 121°C for 15 min. All the cultures were maintained by subculturing in the same medium at 35 days intervals in a growth chamber at 25 ± 2° C under a photoperiod of 16/8h (light/dark) supplied by cool-white fluorescent tubes, giving 60 μmol m^{-2} s^{-1} photosynthetic photon flux. The *in vitro* regenerated plantlets with well developed shoots and roots were washed with distilled water and transferred to plastic cups and then to earthern ware pots (9 cm dia) containing sterilized peat soil and sand (1:1) under diffused 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 12 replicates. The data were analyzed using one way analysis of variance (ANOVA) and means were compared using a Duncan's multiple range test at 0.05 level of significance.

Preliminary phytochmical screening

100 g of leaf and 50 g of calli powder were successively extracted with 250 ml of methanolic solvent for 24 h using soxhlet apparatus. The extracts were evaporated under vacuum in a rotatory evaporator (below 40 º C). The left over powder was considered 100%. The condensed small portion of the dry extract was used for phytochemical screening test. Dragendroffs test for alkaloids, gelatin test for tannin, ferric chloride test for phenolic compounds, Fehlings test for carbohydrates and Libermann- Burchard test for phytosteroids were employed (Harborne,1973;Trease and Evans,1989).

Results and Discussion

The regeneration responses of mature leaf explants without petiole that were placed on MS with BA (1 μM), MS with Kin (1 μM) and in MS supplemented with BA (1μM) + Kin (1μM) are presented in Table 1 and Fig 1. The cultured explants exhibited regeneration responses after 8 days. In MS supplemented with Kin (1 μM) multiple shooting (4.6) and rooting (3.6) was developed in 90% cultures after 25 days. There were less number of shoots (1.2) and roots (3.2) formed by leaf cultures on MS+BA (1μM). The leaf explants placed on MS +Kin (1 μM) +BA (1μM) also produced shoots and roots with a frequency of 2.4 and 4.2 respectively. In all cultures, development of globular somatic cells to organized structures formed at the cut or wounded part of adaxial leaf lamina regions on attached medium. The development of callus and shoot buds directly from leaf explants of *T.*

* Corresponding Author, Email: rathinavel_sn@yahoo.com, Fax: 91452 – 2690505

ISSN: 2218-1768

indica placed on MS with Kin or BA was also reported by Nema *et al.* (2005). The length of shoot (2.4-5.6 cm) and root (5.2-7.4 cm) was also greater in the combinations of MS with cytokinin (Kin or BA) at lower concentrations as compared to other hormonal combinations used in this study. BA at high concentration (4 µM) induced both shooting (2.4) and rooting (3.4) but with intervention of callus in 70% cultures. The similar regeneration responses were also found in the combination of BA (2 µM) +2.4-D (4.5 µM). It evidences that lower concentration of cytokinin (BA) and auxin (2, 4-D) found to be beneficial for induction of shoot buds from leaf explants (Nema *et al.*, 2005). The leaf explants implanted in the combinations of MS + BA (2.5 µM)+2,4-D (3.5 µM) and MS + BA (6µM)+NAA(4 µM) + Ads (10 µM) showed callusing with biomass ranging from 1 to 1.21 g in 80 – 90% of cultures but neither induced shoots nor roots (Table 1& Fig.1). The capacity of auxin and or 2,4-D to induce embryogenic callus has been reported previously in *T. indica* and in several other crops (Rao *et al.,* 1970; Chang, 1991; Joseph *et al.*, 2000). Non-embryogenic callusing from leaf bits of *T. indica* was also noticed in MS media with 2, 4-D, TDZ and BA alone concentrations but the quality and quantity of callus was better in MS with 2, 4-D than other combinations (Chandrasekhar *et al.*, 2006). In the present study, the developed calli subcultured on fresh same medium were compact and white green at the beginning and appeared as globular embryo like structures. But, the transfer of greenish globular structures to fresh MS medium with Kin at 1 µM induced shoot buds and roots after 4 weeks of culture periods. Similarly, Chandrasekhar *et al.* (2006) have developed shoots and roots from somatic embryos derived from leaf explants on placing them in solid MS basal medium without growth regulators. The induction of callus was also observed on the cut edges of leaf explants of *T. indica* on MS supplemented with different concentrations of 2, 4-D and the subculture of callus in the medium with 2,4-D and Kin produced numerous organized structures (Jayanthi and Mandal, 2001). In general, it was observed that the regeneration responses were better in MS with Kin at 1 µM after 4 weeks of cultures as compared to other combinations used in this study. The increase in the concentration of BA and its combination with auxins found to increase poor organogenic responses or production of callus alone in 80-90% of cultures. The regenerants were successfully transferred to ½ MS medium and subsequently to pots for hardening and acclimatization. The survival rate was 62%. The regenerated plantlets were phenotypically similar when compared with wild plants. The phytochemical analysis made in the calli extracted with methanol showed the presence of alkaloids, carbohydrates and steroids and absence of tannins and phenolic compounds as compared to methanolic extract of adult leaf of *T. indica* (Table-2).

Table: 1. *In vitro* plantlets regeneration of *Tylophora indica* from leaf explants

MS Medium supplemented with growth regulators	Days taken for regeneration	% of culture showing				Shoot[a]		Root[a]	
		Callus	Shoot	Root	Callus bio Mass (gm)	No. of shoots	Length (cm)	No. of roots	Length (cm)
MS	0	0	0	0	0	0	0	0	0
MS + KIN (1 µM)	8	0	90	80	0	4.6 ±1.1a	5.6±0.9a	3.6±0.6a b	5.2±1.1b
MS + BA (1 µM)	8	0	70	80	0	1.2±0.2c	2.8±0.8a	3.2±0.8a	7.4±0.5a
MS+ BA (4 µM)	8	70	80	80	0.78	2.4±0.2b	1.4±0.2b	3.4±0.5b	2.2±0.5c
MS + KIN (1 µM)+ BA (1 µM)	8	0	80	90	0	2.4±0.5bc	2.4±0.2b	4.2±1.2a	6.4±1.2a
MS+BA (2.5 µM) + 2.4 D (3.5 µM)	5	80	0	0	1.00	-	-	-	-
MS+BA (2 µM) + 2.4 D (4.5 µM)	7	80	80	80-	0.41	2.0±0.3b	.4±0.2bc	3.2±0.8b	2.2±0.3c
MS + BA (6 µM) + NAA (4 µM) + Ads(10 µM)	8	90	0	0	1.21	-	-	-	-

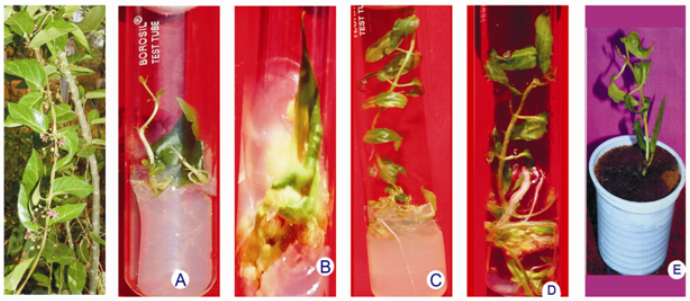
[a] values represent means ± SE. Means followed by the same letter within columns are not significantly different (p = 0.05) using Duncan's multiple range test

Table: 2. Phytochemical screening of methanolic leaf and calli extracts of *T. indica*

Phytoconstituents	Leaf	Calli
Alkaloids	+	+
Taninns	+	-
Phenolic compounds	+	-
Steroids	+	+
Carbohydrates	+	+

+ Indicates presence of compounds: - Indicates absence of compounds

Fig 1:In vitro regeneration of Tylophora indica from leaf explants



A.Shoot Multiplication in MS+KIN(1µM) B.Callusing inMS+BA(2.5µM) +2.4,D(3.5µM)
C.Rooting in MS+BA(1µM) D.Root hardening in 1/2 MS medium E.Potted plant

Similarly, the presence of alkaloids, carbohydrates, steroids and tannins was confirmed while analysing the alcoholic and aqueous extracts of root (Patel *et al.*, 2006) and leaf (Gujrati *et al.*, 2007) of *T. indica.* However, further studies on isolation and characterization of bioactive compounds from callus cultures are suggested for potential utility of *in vitro* technology for pharmaceutical purpose.

It is concluded that the present study provides a simple protocol for shoot and root induction from mature leaf explants of *T. indica* for clonal propagation. The leaf explants had considerable morphogenetic potential in MS media with low concentration of Kin or BA alone and or in combination of Kin with BA. It may be interpreted that shoot and root inducing characteristics in cultures would be associated with hormonal type and combinations in culture medium as reported in the earlier studies (Jaiwal and Gulati, 1999; Chattopadhyay *et al.*, 1995). Further standardization of medium and or selection of other explants are under progress to develop an efficient *in vitro* propagation system of the medicinal plant *T. indica* which can be successfully mass produced, thereby providing a source for the extraction of drugs and also ensuring conservation of this valuable species in nature.

Acknowledgement

Authors are gratefully acknowledged the University Grants Commission (UGC), New Delhi for financial assistance.

References

Bera, T.K. and Roy, S.C. (1993). Micropropagation of *Tylophora indica* (Burm. f.) Merrill. by multiple bud formation from mature leaf explants without callus intervention. Bot. Bull. Acad. Sci. 34, 83-87.

Chandrasekhar, T., Hussain, T.M., Rama Gopal, G., and Srinivasa Rao, J.V. (2006). Somatic embryogenesis of *Tylophora indica* (Burm.f.) Merrill., an important medicinal plant. International J. of Applied Science and Engineering. 4 (1), 33-40.

Chang, W.C., and Bamboo, S. (1991). In: Bajaj YPS, ed. Biotechnology in agriculture and forestry, Vol.16. Berlin: Springer-Verlag; 211-237.

Chattopadhyay, S., Datta, S.K., and Mahato, S.B. (1995). Rapid micropropagation of *Mucuna pruriens f. pruriens* L. Plant Cell Rep. 15, 271- 260.

Faisal, M., and Anis, M. (2005). An efficient *in vitro* method for mass propagation of *Tylophora indica*. Biologia Plantarum. 49(2), 257-260.

Faisal, M., Sindh, S., and Anis, M. (2006). *In vitro* regeneration and plant establishment of *Tylophora indica* (Burm. f.) Merrill: petiole callus culture. In vitro Cell. and Dev. Biol. Plant. 41(4), 511-515.

Gujrati, V., Patel, N., Rao, V.N., Nandakumar, K., Gouda, T.S., and Shanta Kumar, S.M. (2007). Hepatoprotective activity of alcoholic and aqueous extracts of leaves of *Tylophora indica* (Lin.) in rats. Indian J. Pharmacol. 39,43-47.

Harborne, J.B. (1973). Phytochemical methods, A guide to modern technique of plant analysis, Chapman and Hall, London.

Jaiwal, P.K., and Gulati, A. (1999). *In vitro* high frequency plant regeneration of a tree legume *Tamarindus indica* L. Plant Cell Rep. 10, 565 - 573.

Jayanthi, M., and Mandal, P.K. (2001). Plant regeneration through somatic embryogenesis and RAPD analysis of regenerative plants in *Tylophora indica* (Burm.f.) Merrill. In Vitro Cell. Dev. Biol. Plant. 37,576-580.

Joseph, T., Yeoh, H.H., and Loh, C.S. (2000). Somatic embryogenesis, plant regeneration and cryogenesis of *Manihot glaziovii* Muell.Agr. (Ceara rubber). Plant Cell Rep.19, 535-538.

Manjula, S., Job, A., and Nair, G.M. (2000). Somatic embryogenesis from leaf derived callus of *Tylophora indica* (Burm. f.) Merrill. Indian J. Exp. Biol. 38(10), 1069-1072.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays for tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Nema, R.K., Ramawat, K.G., Gupta, G.D., Tanwar, Y.S., and Meeta, M. (2005). Rapid micropropagation of *Tylophora indica*. Phcog. Mac. 52-55.

Patel, N.J., Gujarati, V.B., Gouda, T.S., Venkat Rao, N., Nandakumar, K., and Shantakumar, S.M. (2006). Antidiarrhoeal activity of alcoholic and aqueous extract of roots of *Tylophora indica* (Wight & Arn.) in rodents. Pharmacologyonline.1, 19-29.

Rao, P.S., Narayanaswami, S., and Benjamin, B.D. (1970). Differentiation ex-ovule of embryos and plantlets in stem tissue cultures of *Tylophora indica*. Physiol. Plant. 23, 140-144.

Trease, G.E., and Evans, W.C. (1989). "A text- book of pharmacognosy" Academic Press, London. 22-40.