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Research Article

PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS IN *TYLOPHORA INDICA* (BURM. F.) MERRILL -AN ENDANGERED MEDICINAL CLIMBER

[*]Vineet Soni[1], Megha Bhushan[2] and P.L. Swarnkar[3]
[1]Plant Bioenergetics and Biotechnology Laboratory, Department of Botany, Mohanlal Sukhadia University, Udaipur-313001, Rajasthan, India
[2]School of Life Science, Jaipur National University, Jaipur-302025, Rajasthan, India
[3]Department of Botany, University of Rajasthan, Jaipur, India
[*]Author for Correspondence

ABSTRACT
A effective protocol for plant regeneration via somatic embryogenesis has been developed for *Tylophora indica* L. Embryogenic callus was initiated from stem explants on Murashige and Skoog (MS) medium supplemented with 2 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1}6-benzylaminopurine (BAP). The calli showed highest frequency of embryogenesis (89.04 ± 3.67) and number of somatic embryos per culture (72.66 ± 3.51) on MS medium fortified with BAP (5.0 mg l^{-1}) and L-glutamine (200 mg l^{-1}). Maximum percent germination (78.0±3.2) of somatic embryos was observed on ½ MS devoid of plant growth regulators. Well developed plantlets with high photosynthetic performance ($\varphi_{P0=}$ Fv/Fm ≥ 0.8) were successfully shifted to natural conditions. The overall survival rate from *in vitro* growth to field transfer was 74 %. The developed protocol can be successfully used for large-scale multiplication and conservation this medicinal plant species.

Keywords: *Tylophora Indica, Micropropagation, Somatic Embryogenesis, Plant Growth Regulators*

INTRODUCTION
Tylophora indica (Burm. F.) Merrill. (Asclepiadaceae), a perennial climber, is used in the Indian and Chinese traditional system of medicine since prehistoric time for the treatment of asthma, bronchitis, whooping cough, dysentery and diarrhoea.
The pharmacological activity of this plant is mainly due to the presence of alkaloid tylophorine, which exerts anti-asthamatic (Gupta *et al.*, 1979), anti-inflammatory (Gopalakrishnan *et al.*, 1980); anti-angiogenic and anti-tumor activity (Saraswati *et al.*, 2013). The plant also possesses anti-allergic (Nayampalli and Sheth, 1979), anti-ameobicidal (Bhutani *et al.*, 1987), immunomodulatory (Ganguly *et al.*, 2001), anti-viral (Xi *et al.*, 2006), hepatoprotective (Gujrati *et al.*, 2007; Mujeeb *et al.*, 2009), and diuretic (Meera *et al.*, 2009) activities. Unfortunately, the wild population of this plant is now threatened into extinction because of uncontrolled harvesting, over exploitation and lack of organized cultivation (Thomas and Philip, 2005).
Conventionally *T. indica* is propagated mainly through the seeds and stem cuttings. However, germination of seeds is poor and propagation through stem cuttings solely relies on season for multiplication, which makes it an ineffective way for the conservation of this medicinally important plant (Chandrasekhar *et al.*, 2006).
So, this plant species requires urgent attention for its protection, large scale systematic cultivation and conservation.
The establishment of *in vitro* regeneration protocol will be an important action for multiplication, and germplasm conservation of *T. indica*. During past years, considerable efforts have been made for *in vitro* regeneration of *T. indica* through induction of somatic embryogenesis via leaf explants (Jayanthi and Mandal, 2001; Chandra Sekhar *et al.*, 2006; Sahai *et al.*, 2010), shoot organogenesis (Faisal and Anis, 2005; Thomas and Philip, 2005), nodal segment culture (Faisal *et al.*, 2007). The present communication reports the protocol for the plant regeneration via somatic embryogenesis of *T. indica* using stem callus *in vitro*.

MATERIALS AND METHODS

Plant Material and Surface Sterilization: The young shoots of *T. indica* were collected from plants growing at the University`s Botanical Garden. The stem segments dissected and washed thoroughly under running tap water for 30 min to eliminate dust particles and then with 5% teepol for 8 -10 min and rinsed several times in sterile distilled water. Then, the explants were treated with an antifungal agent (Bavistin) for 1 h and the again rinsed three times with sterile distilled water. Thereafter, the explants were surface sterilized under a laminar flow chamber with aqueous solution of 0.1% (w/v) solution of $HgCl_2$ for 2 min and finally washed with sterile distilled water for 2–3 times.

Culture Media and Growth Conditions: After trimming both the cut ends, the sterilized stem explants were inoculated vertically on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and various concentrations (0.5 – 5.0 mg l^{-1}) of 2,4-D. The efficiency of the optimal concentration of 2, 4-D with different concentrations of BAP (0.5 – 5.0 mg l^{-1}) was also evaluated for high frequency induction of embryogenic callus. MS medium lacking plant growth regulators served as control.

For the induction of somatic embryogenesis, embryogenic calli were subcultured on MS medium containing BAP (0.5-5.0 mg l^{-1}) either alone or combination with L-glutamine (50-500 mgl^{-1}).

The cultures were kept in a growth chamber at 25 ± 2 °C under a 16/8 h photoperiod conditions using a photosynthetic photon flux density (PPFD) of 40 µmol/m^2s^1 provided by cool white fluorescent tubes (Philips, India). After 4 weeks of culture, the percentage of callus that initiated somatic embryos and the number of embryos were calculated. Fully developed somatic embryos were carefully isolated from callus cultures and transferred to solid MS medium without growth regulators for conversion to complete plantlets.

Hardening and Acclimatization: Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic cups (10 cm × 8 cm) containing sterilized mixture of sterile soil, sand and coco peat (1:2:1). The plantlets covered in transparent polyethylene bags were kept for 4 weeks in growth chamber at 25 ± 2 °C with 16 h photoperiod and 40 µmol/m^2 s^1 of irradiation. The plantlets were irrigated with ½ MS for two weeks. φ_{P0} of plantlets growing under growth chamber and green house conditions was regularly measured using a Plant Efficiency Analyser, PEA (Hansatech Instruments, Kings Lynn, Norfolk, U.K.) according to Heber *et al.*, (2011).

The leaf samples were dark adapted for 2 h before the fluorescence measurements. The plants with high φ_{P0} ($\geq$ 0.8) were subsequently transferred to large pots containing normal garden soil and were maintained in natural conditions. The survival rate of plantlets was recorded after 1 month of transfer to natural conditions.

Experimental Design and Statistical Analysis: In the present study, 50 explants were used for each combination of different hormones supplemented with MS medium under similar conditions. Data are represent the mean ± SE of 3 independent explants (n = 3).

RESULTS AND DISCUSSION

Induction of Embryogenic Callus: All concentrations of 2.4-D (0.5-5.0 mgl^{-1}) induced callus formation at the basal ends of stem explants of *T. indica*. Optimum callus induction was recorded on MS medium enriched with 2,4-D (2.0 mgl^{-1}) wherein 61±1.17 % explants produced yellowish green, friable and embryogenic callus cultures. Further increasing the 2,4-D concentration showed induction of compact and non-embryogenic callus (Figure 1A). High frequency induction of friable embryogenic callus (88.40±2.37 %) was achieved on MS supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP (Table 1). Growth of the callus on MS medium amended with 2,4-D (2 mg l^{-1}) and BAP (0.5 mg l^{-1}) was slow at the beginning, but by the end of the second subculture onto the same medium, it produced green embryogenic callus (Figure 1B). Repeated subculture for more than 6 weeks on callus induction medium resulted in hyper-hydration of cultures and further proliferation of calli ceased. Hormone free medium could not elicit any response for callus initiation, hence, not included in data analysis. Embryogenic callus obtained

from above mentioned medium was used to investigate the influence of BAP and L-glutamine on the induction of somatic embryogenesis.

In most cases, auxins mainly 2, 4-D is required for the induction of embryogenic callus leading to somatic embryogenesis (Tangolar *et al.,* 2008; Vengadesan and Pijut, 2009; Chen *et al.,* 2010). On the other hand, it was also reported that 2, 4-D individually could not produce adequate number of somatic embryos and addition of cytokinin in 2, 4-D containing medium was beneficial for somatic embryogenesis (Shu *et al.,* 2005; Das, 2011). This type of various morphogenetic responses to different combination of plant growth regulators may be due to differences in genetic make-up among different plant species (Xie and Hong, 2001).

Somatic Embryogenesis: Clusters of globular and heart-shaped embryos per explant were produced within 3 weeks by culturing embryogenic callus on MS medium augmented with 3% sucrose and BAP (5.0 mg l^{-1}). Addition of various concentrations of L-glutamine (50-500mg l^{-1}) along with optimal concentration of BAP (5.0 mg l^{-1}) in MS medium significantly enhanced the frequency of somatic embryogenesis (Table 2). The highest frequency of embryogenesis (89.04 ± 3.67) and number of somatic embryos per culture (72.66 ± 3.51) was achieved on MS medium containing 5.0 mg l^{-1} BAP and 200 mgl^{-1}L-glutamine (Figure 1C). Somatic embryos underwent maturation and germination when kept for longer duration in the same medium. Maximum percent germination (78.0±3.2) of somatic embryos was recorded on plant growth regulator free ½ MS (Figure 1 D, E).

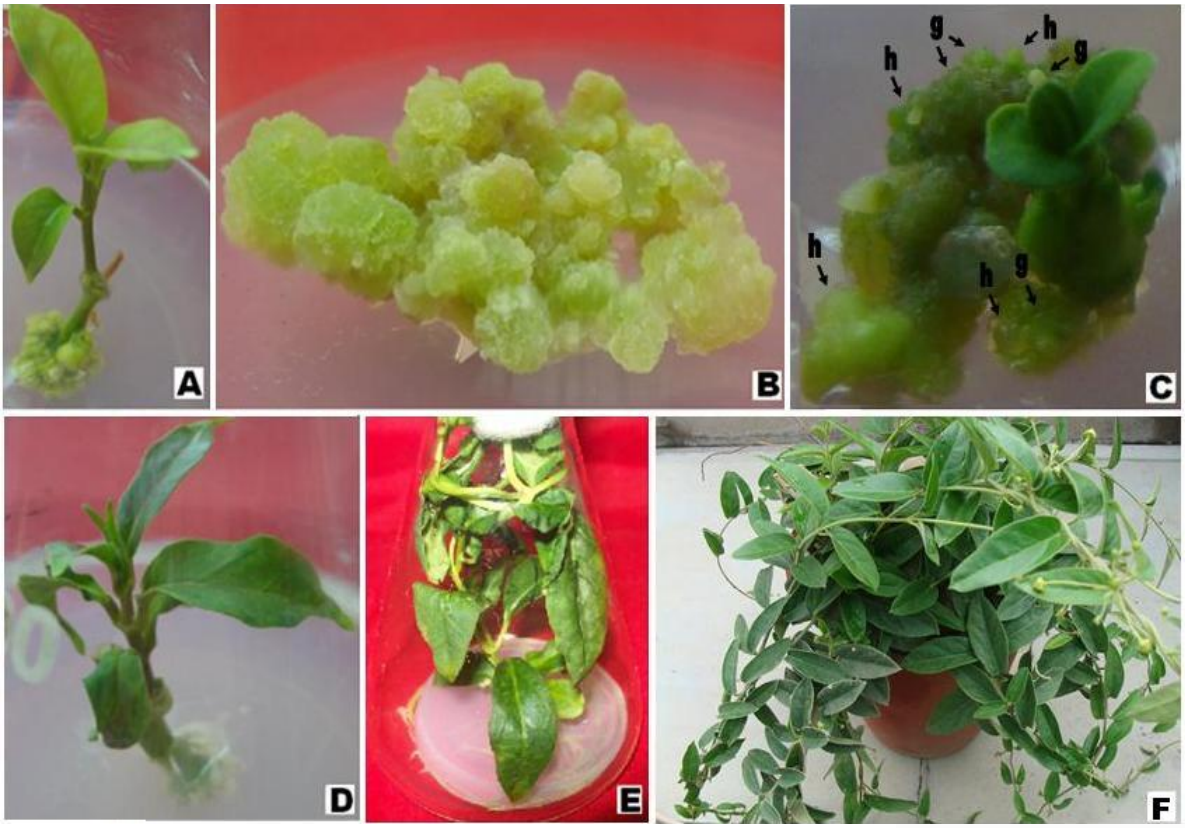

Figure 1: Somatic Embryogenesis in *T. indica;* (A) Induction of Compact Non-Embryogenic Callus on MS Medium Containing 2,4-D (5.0 mg l^{-1}); (B) Induced Embryogenic Callus on MS Medium Containing 2,4-D (2 mg l^{-1}) and BAP (0.5 mg l^{-1}); (C) Induction of Somatic Embryogenesis and the Development of Globular (g) and Heart (h)-Shaped Embryos on MS Medium Containing BAP (5.0 mg l^{-1}) and L-Glutamine (200 mg l^{-1}); (D, E) Plantlet Formation from Somatic Embryos on Plant Growth Regulator Free Half-Strength MS Medium; (F) Establishment of *In Vitro* Grown Plantlet under Natural Condition

Our studies further revealed that combination of BAP and L-glutamine is essential for the somatic embryogenesis in *T. indica*. The frequency of somatic embryogenesis enhanced significantly when embryogenic callus was cultured on medium supplemented with L-glutamine and BAP than those cultured on BAP alone. Earlier, the beneficial role of L-glutamine in the induction of somatic embryogenesis has also been reported in a number of studies (Sahrawat and Chand, 2011; Rathore *et al.*, 2012, Kumar *et al.,* 2015).

Table 1: Influence of 2, 4-D and BAP on Embryogenic Callus Induction after 4 Weeks of Culture of Stem Explants of *T. Indica*, Values Represent the Mean ± SE of 3 Independent Explants (n = 3)

Plant Growth Regulators (mg l^{-1})		Percentage of Explant Induced Embryogenic Callus
2,4-D	BAP	
0.5	0.0	22.01 ± 1.21
1.0	0.0	37.43 ± 1.63
2.0	0.0	41.00 ± 1.17
3.0	0.0	33.27 ± 1.05
4.0	0.0	14.94 ± 1.71
5.0	0.0	8.13 ± 0.56
2.0	0.5	88.40 ± 2.37
2.0	1.0	70.61 ± 2.78
2.0	2.0	59.23 ± 1.60
2.0	3.0	52.48 ± 2.10
2.0	4.0	11.50 ± 1.06
2.0	5.0	04.84 ± 0.91

Table 2: Influence of BAP and L-Glutamine on the Induction of Somatic Embryogenesis after 4 Weeks of Culture, Values Represent the Mean ± SE of 3 Independent Explants (n = 3)

Treatments	Frequency of Embryogenesis (%)	Number of Somatic Embryos/Culture
0.5 mg l^{-1} BAP	1.93± 0.32	2.03± 0.41
1.0 mg l^{-1} BAP	4.02± 0.46	2.55± 0.42
2.0 mg l^{-1} BAP	4.63± 0.31	4.10± 0.53
3.0 mg l^{-1} BAP	16.45 ± 1.41	9.14 ± 1.47
4.0 mg l^{-1} BAP	36.0 ± 2.64	12.58 ± 2.35
5.0 mg l^{-1} BAP	44.47 ± 3.80	56.50 ± 1.10
5.0 mg l^{-1} BAP + 50 mg l^{-1}L-glutamine	67.23 ± 2.33	28.50 ± 3.44
5.0 mg l^{-1} BAP + 100 mg l^{-1}L-glutamine	71.92 ± 4.37	32.66 ± 2.95
5.0 mg l^{-1} BAP + 200 mg l^{-1}L-glutamine	89.04 ± 3.67	72.66 ± 3.51
5.0 mg l^{-1} BAP + 300 mg l^{-1}L-glutamine	61.01 ± 2.61	32.66 ± 2.59
5.0 mg l^{-1} BAP + 400 mg l^{-1}L-glutamine	58.72 ± 1.66	24.66 ± 2.57

Hardening and Acclimatization: Plantlets regenerated *in vitro* were successfully acclimatized in the growth chamber (79 % survival) and then in the greenhouse (81 % survival). To ensure high survival rate in natural conditions, φ_{P0} of *in vitro* raised plantlets were regularly measured during hardening and acclimatization process. Plantlets derived from somatic-embryos significantly achieved φ_{P0} value from 0.37 to 0.83 within 6 weeks of hardening and acclimatization process in growth chamber and green house conditions. Fully acclimatized plants having high φ_{P0} ($\geq$ 0.8) were shifted and maintained under natural conditions with 100% survival rate.

The large scale production of micropropagated plantlets is often limited by poor survival when plantlets are transferred in *ex vitro* conditions. The high exogenous sucrose content in the medium has been shown to inhibit photosynthetic gene expression, leading to low photosynthetic rates (Fuentes *et al.,* 2005). In the

present study, φ_{P0} increased throughout hardening and acclimatization process, providing evidence for increasing photosynthetic performance. Acclimatized plantlets with high φ_{P0} ($\geq$ 0.8) were successfully established under natural conditions. The developed *in vitro* protocol can be useful for the conservation and large scale propagation of this endangered plant species. Photosynthetic screening of *in vitro* raised plantlets, as described in present study, can also be used for achieving high survival rate of *in vitro* raised plantlets in the field.

Abbreviations

φ_{P0}- maximum quantum yield of primary photochemistry; BAP- 6-benzylaminopurine; 2,4-D- 2,4-dichlorophenoxyacetic acid; Fv- variable fluorescence; Fm- maximum fluorescence; MS-Murashige and Skoog; ½ MS - medium containing half-strength Murashige and Skoog micro- and macro-nutrients

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