

## In vitro propagation of *Psoralea corylifolia* L. by somatic embryogenesis in cell suspension culture

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**Abstract** An effective protocol was developed for in vitro propagation of *Psoralea corylifolia* via somatic embryogenesis in cell suspension culture. Embryogenic callus was obtained on Murashige and Skoog (MS) medium supplemented with 6  $\mu\text{M}$  naphthaleneacetic acid (NAA) and 30  $\mu\text{M}$  glutamine from transverse TCLs from 10-day-old hypocotyl explants with a 96.4% frequency. Embryogenic callus produced a higher number of somatic embryos ( $123.7 \pm 1.24$  per gram fresh weight callus) on MS medium containing 30  $\text{g l}^{-1}$  sucrose, 1  $\mu\text{M}$  NAA, 4  $\mu\text{M}$  benzyladenine (BA), 15  $\mu\text{M}$  glutamine and 2  $\mu\text{M}$  abscisic acid (ABA) after 4 weeks of culture. Somatic embryos successfully germinated (97.6%) on  $\frac{1}{2}$  MS medium containing 20  $\text{g l}^{-1}$  sucrose, 8  $\text{g l}^{-1}$  agar and supplemented with 2  $\mu\text{M}$  BA, 1  $\mu\text{M}$  ABA and 2  $\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ) within 2 weeks of culture. Somatic embryos developed into normal plants, which hardened with 100% efficiency in soil in a growth chamber. Plants were successfully transferred to greenhouse and subsequently established in the field. Plant survival percentage in the field differed with seasonal variations. Average psoralen content of 12.9  $\mu\text{g g}^{-1}$  DW was measured in different stages of somatic embryo development by high-performance liquid chromatography

(HPLC). This protocol will be helpful for efficient propagation of elite clones on a mass scale, conservation efforts of this species and for secondary metabolites production studies.

**Keywords** Growth regulators · Hypocotyl · Indirect embryogenesis · *Psoralea corylifolia* · Psoralen · tTCLs

### Introduction

*Psoralea corylifolia* L. (Bhavanchi, *Fabaceae*) is an endangered medicinal plant distributed widely in tropical and sub-tropical regions. It is an attractive annual herb producing with bluish purple flowers. The plant contains psoralen, an important compound which is commercially valuable. Psoralen possesses photosensitizing, photobiological and phototherapeutic properties used for the photochemotherapy of vitiligo and skin diseases such as psoriasis, mycosis fungoides and eczema (Frank et al. 1998). The plant exhibits antitumour, antibacterial, antifungal and antioxidative activities. It is also used in curing stomach ache, antihelmintic, diuretic and diaphoretic in febrile conditions. Many Indian pharmaceutical industries have used *P. corylifolia* as a raw material in the production of medicines and ayurvedic skin care soap (Baskaran and Jayabalan 2007). This medicinally important plant suffers from high seedling mortality and from very fast declined of wild populations due to indiscriminate, illegal collections and of habitat destruction. Therefore, this species is included in the endangered list (Baskaran and Jayabalan 2008). Hence, there is a need to develop an appropriate protocol for mass propagation and conservation to satisfy the pharmaceutical demand.

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In vitro culture techniques provide a means of rapid plant propagation and a tool for crop improvement. Through tTCLs technology, micropropagation could be obtained in recalcitrant (Mulin and Bellio-Spataru 2000; Leguillon et al. 2003) medicinal (Ahn et al. 1996) and other crop plant species (Baskaran et al. 2006; Ben Ghnaya et al. 2008) in rapid multiplication. Moreover, tissue culture technology provides means of mass propagation of a species of interest with profound potential economic spin-offs (Teixeira da Silva 2003). Somatic embryogenesis by cell suspension culture offers an in vitro method for mass propagation of important medicinal plant species. In addition, somatic embryogenesis enables to produce large numbers of plantlets (Martin 2004) and bioactive compounds within a short span of time (Aderkas et al. 2002; Jeong et al. 2005). The technique is also suitable for germplasm conservation, the selection of genetic variants for desirable characters, the generation of somaclonal variants and for performing cellular genetic manipulations (Vasil 1988). Therefore, somatic embryogenesis could play an important role in *P. corylifolia* including fulfilling the pharmaceutical demands. Although studies on somatic embryogenesis of *P. corylifolia* have been published previously (Sahrawat and Chand 2001; Chand and Sahrawat 2002, 2007), the present investigation describes for the first time the possibility to obtain rapid and higher numbers of somatic embryos by mass propagation in cell suspension culture.

The objective of the present investigation was aimed at developing efficient plant regeneration systems via indirect (through callus stage) embryogenesis from hypocotyl-derived TCLs explants in cell suspension culture, which can be utilized for conservation to create variation and multiply the *P. corylifolia* plants in vitro. The present study reports on successful embryogenesis and plant regeneration protocol from tTCLs hypocotyl explants by evaluating various carbon sources, different concentrations and combinations of plant growth regulators for embryo production, and also to investigate the psoralen content in somatic embryos (at different developmental stages) of *P. corylifolia*.

## Materials and methods

### Explant preparation and embryogenic callus induction

Ten-day-old hypocotyl explants of *P. corylifolia* were collected from medicinal plant garden of the Bharathidasan University, Tiruchirappalli, India. Hypocotyl explants were surface sterilized with a solution containing commercial detergent Teepol (Reckitt Benckiser, India) for 2 min and aqueous mercuric chloride (0.1% w/v) for 3 min. The

explants were washed five times with sterile distilled water.  $10 \times 0.5$  mm longitudinally thin cell layers (ITCLs) and  $5 \times 1.0$  mm transverse thin cell layers (tTCLs) from hypocotyls were excised under a laminar flow cabinet. For production of embryogenic callus, TCLs were inoculated onto MS (Murashige and Skoog 1962) medium containing  $30 \text{ g l}^{-1}$  sucrose and  $8 \text{ g l}^{-1}$  agar (Agar Type I, Himedia, India), and supplemented with 2–10  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) or 2–10  $\mu\text{M}$  naphthaleneacetic acid (NAA) or 8  $\mu\text{M}$  2,4-D plus 10–50  $\mu\text{M}$  glutamine, or 6  $\mu\text{M}$  NAA plus 10–50  $\mu\text{M}$  glutamine. MS medium devoid of growth regulators was used as control. The media were adjusted to  $\text{pH } 5.8 \pm 0.1$  with 0.1 N NaOH or 0.1 N HCl before gelling with  $8 \text{ g l}^{-1}$  agar. The cultures were maintained at  $25 \pm 2^\circ\text{C}$  under a 16/8 h light/dark regime, under a photon flux density of  $45\text{--}50 \mu\text{mol m}^{-2} \text{ s}^{-1}$  provided by cool white fluorescent tubes (40 W; Phillips, India). The relative humidity (RH) within culture room was maintained at  $55 \pm 5\%$ .

### Development and germination of somatic embryos from suspension culture

For somatic embryos production, 2-week-old white and friable embryogenic calli derived from tTCLs (approximately, 1.0 g fresh weight) were transferred to 250-ml Erlenmeyer flasks (Borosil, India) containing 50 ml of MS liquid medium supplemented with 1  $\mu\text{M}$  NAA, 4  $\mu\text{M}$  benzyladenine (BA) and 10–50  $\text{g l}^{-1}$  glucose or fructose or sucrose or maltose; or 1  $\mu\text{M}$  NAA, 4  $\mu\text{M}$  BA, 30  $\text{g l}^{-1}$  sucrose and 5–25  $\mu\text{M}$  glutamine alone and 1–5  $\mu\text{M}$  abscisic acid (ABA) alone or a combination of glutamine plus ABA. MS medium lacking growth regulators served as control. Cultures were agitated on an orbital shaker (100–110 rpm) under  $12 \mu\text{mol m}^{-2} \text{ s}^{-1}$  cool white fluorescent light with a 16-h photoperiod at  $25 \pm 2^\circ\text{C}$ . After 10 days of culture, embryogenic cell clumps were filtered using a 200- $\mu\text{m}$  stainless steel sieve to remove the large cell clumps. Aliquots of suspension culture (2–10% [v/v]) were transferred to 50 ml of fresh liquid medium of the same composition for subculture. Further three subcultures were carried out with fresh medium of the same composition at 5–7 days intervals for further proliferation. Growth of suspension cultures was monitored by cell counting using a haemocytometer (after extensive resuspension) and ascertaining the packed cell volume (PCV). PCV was determined by centrifuging 5 ml of the suspension at 200g for 5 min. Different developmental stages of embryos (globular, heart and torpedo) were induced after 4 weeks of culture. The number of embryos per flask of culture was determined. All stages of embryos were photographed under a Magnus Stereo Microscope (MSB type; Grand VCD 2000 plus; SWH 10X; transmitted light-FL). The

torpedo embryos were selected from the suspension cultures, and the remaining embryos (globular and heart) were subcultured in the same composition of the medium. For germination and plantlet formation, torpedo embryos were transferred to  $\frac{1}{2}$  MS medium containing  $20 \text{ g l}^{-1}$  sucrose and  $8 \text{ g l}^{-1}$  agar and supplemented with  $1\text{--}4 \text{ }\mu\text{M}$  BA,  $0.5\text{--}2 \text{ }\mu\text{M}$  ABA and  $1\text{--}4 \text{ }\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ) either individually or in combination. The medium lacking growth regulators served as control. The cultures were incubated at  $25 \pm 2^\circ\text{C}$  under a 16-h photoperiod.

#### Transplantation

Regenerated plantlets with healthy shoot and root systems were transferred to plastic pots containing soil mixture and vermiculite ( $150 \text{ g/pot}$ ) (3:1v/v) and irrigated with 50 ml of tap water used as control, or 50 ml of  $\frac{1}{2}$  MS basal salt solution supplemented with  $5 \text{ }\mu\text{M}$  indole-3-butyric acid (IBA) and  $100 \text{ mg l}^{-1}$  Bavistin® (BVN), respectively, used to enhance the root formation and prevent the fungal contamination. The plantlets were covered with transparent polythene bags to prevent desiccation and were maintained in a growth chamber (Sanyo, Japan). The relative humidity was reduced gradually, and after 1 month, plants were transferred to pots filled with 2:1 (v/v) mixture of soil and organic manure and maintained in a greenhouse. After 2 months, the plants were established in the field. The survival percentage was evaluated in summer (March–June) and winter (September–December) seasons.

#### Estimation of psoralen

After 4 weeks, different developmental stages of somatic embryos (globular, heart and torpedo) of *P. corylifolia* were evaluated for psoralen content by high-performance liquid chromatography (HPLC). The somatic embryos were oven-dried at  $45 \pm 2^\circ\text{C}$  for 30 min. The dried tissues ( $1.0 \text{ g}$ ) were ground to a powder and soaked overnight in a 100% HPLC grade methanol (10 ml). The mixture was filtered through Whatman No.1 filter paper, and residue was redissolved in methanol (5 ml), filtered through  $0.45 \text{ }\mu\text{m}$  nylon filter (Millipore Corporation, USA) and centrifuged at 1,200 rpm for 5 min. The extract containing psoralen was analysed by HPLC on a GEMINI (Phenomenex) reverse phase C-18 ( $5.0 \text{ }\mu\text{m}$  particle size)  $250 \times 4.6 \text{ mm}$  column. The mobile phase was methanol/water (50:50 v/v) mixture supplied at the rate of  $0.8 \text{ ml/min}$ , using a constant temperature of  $20^\circ\text{C}$  during the analysis. The psoralen was detected by monitoring absorbance at 254 nm using a UV detector (SHIMADZU-Prominence model). Duplicate injections ( $20 \text{ }\mu\text{l}$ ) from sample were compared with the standard for quantification.

Standard psoralen was purchased from Sigma Chemical, Mumbai, India. The experiments were repeated thrice. The amount of psoralen was calculated by the external standard method of quantification (Scott 1996).

#### Data collection and statistical analysis

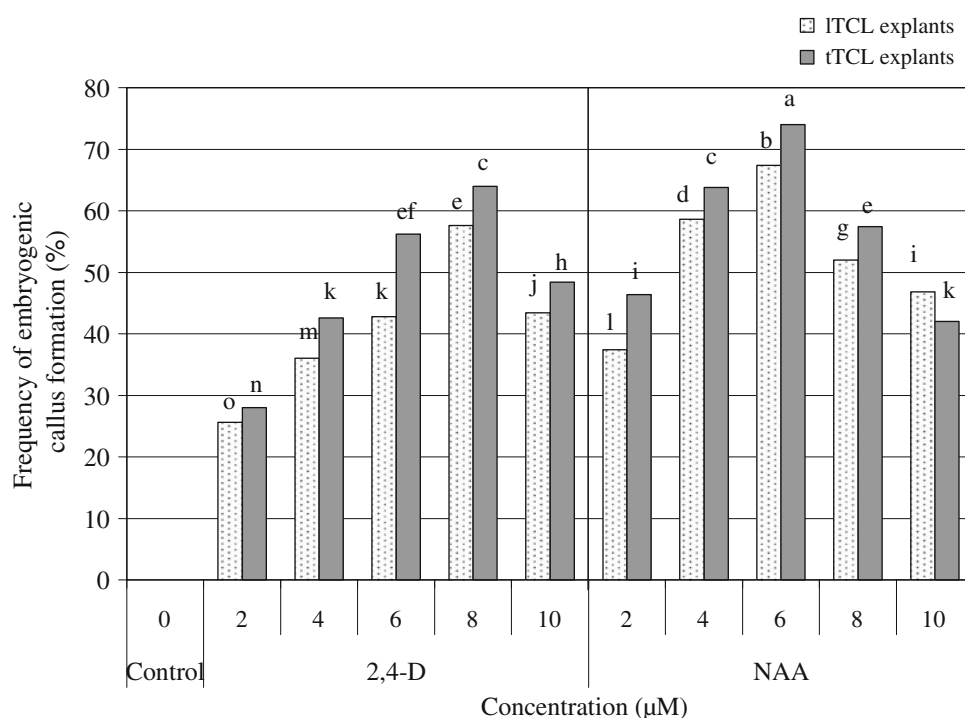
The data were collected after 2 weeks for frequency of embryogenic callus formation (%), 4 weeks for number of somatic embryos and additional 2 weeks for frequency of germination (%) experiments. All experiments were repeated three times with 50 explants per treatment. The statistical significance of differences among the means was carried out using Duncan's multiple-range test at 5% level (STATISTICA version 6.0).

## Results and discussion

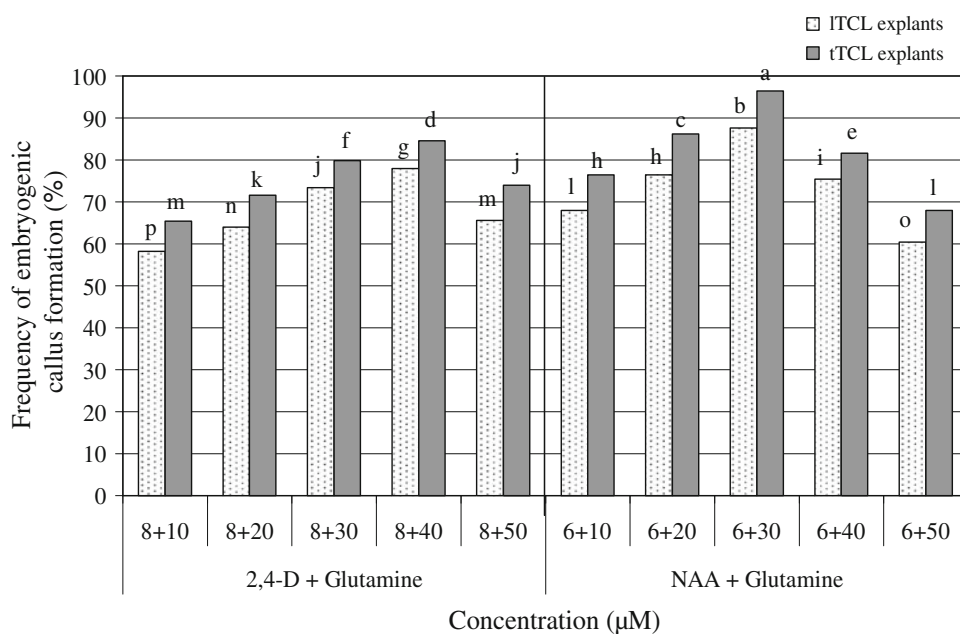
#### Embryogenic callus induction

Auxins (2,4-D and NAA) and glutamine were used to test their effect on induction of embryogenic callus from hypocotyl TCLs explants (Figs. 1, 2). Whitish friable embryogenic callus was obtained in both TCL explants (Fig. 3a, b). Puigderrajols et al. (2001) documented that callus initially showed highly vacuolated cells and later certain cells of these calli become embryogenic, containing dense cytoplasm, small vacuoles, large nuclei and the presence of large amounts of lipids and starch. It is reported that a single cell divides to form few-celled pro-embryo structures which are referred to as embryogenic units. Moreover, recurrent systems are especially suited to the continuous production of friable callus, which is a valuable source of single cells with embryogenic capacity (Fransz and Schel 1991). In our studies, concentrations of 2,4-D or NAA alone produced different frequency of embryogenic callus from both ITCL and tTCL explants while control did not produce embryogenic callus (Fig. 1). However, 2,4-D or NAA combination with glutamine considerably increased frequency of somatic embryogenic callus from both explants (Fig. 2). Similar result was also reported in *Macrotyloma unifloram* by Shamsudeen Varisai et al. (2004). These results suggest that glutamine plays a major role in somatic embryogenesis in *P. corylifolia*; however, the mechanism needs to be investigated. Lower rate of frequency was observed in ITCL explants on MS medium supplemented with  $2 \text{ }\mu\text{M}$  2,4-D (Fig. 1). However, higher rate of frequency of whitish friable embryogenic calli was obtained in tTCL explants on MS medium supplemented with  $6 \text{ }\mu\text{M}$  NAA and  $30 \text{ }\mu\text{M}$  glutamine (Fig. 2). Therefore, tTCLs explants possibly influence rate of callus proliferation due to different types of tissue than

**Fig. 1** Influence of 2,4-D or NAA on embryogenic callus formation on TCL hypocotyl explants of *P. corylifolia*. The results are expressed as the means of three repeat experiments. Different letters indicate significant difference between means ( $P < 0.05$ ); comparison by DMRT. The data were recorded after 2 weeks of culture



**Fig. 2** Influence of different glutamine concentrations added to media with 2,4-D or NAA on embryogenic callus formation on TCL hypocotyl explants of *P. corylifolia*. The results are expressed as the means of three repeat experiments. Different letters indicate significant difference between means ( $P < 0.05$ ); comparison by DMRT. The data were recorded after 2 weeks of culture



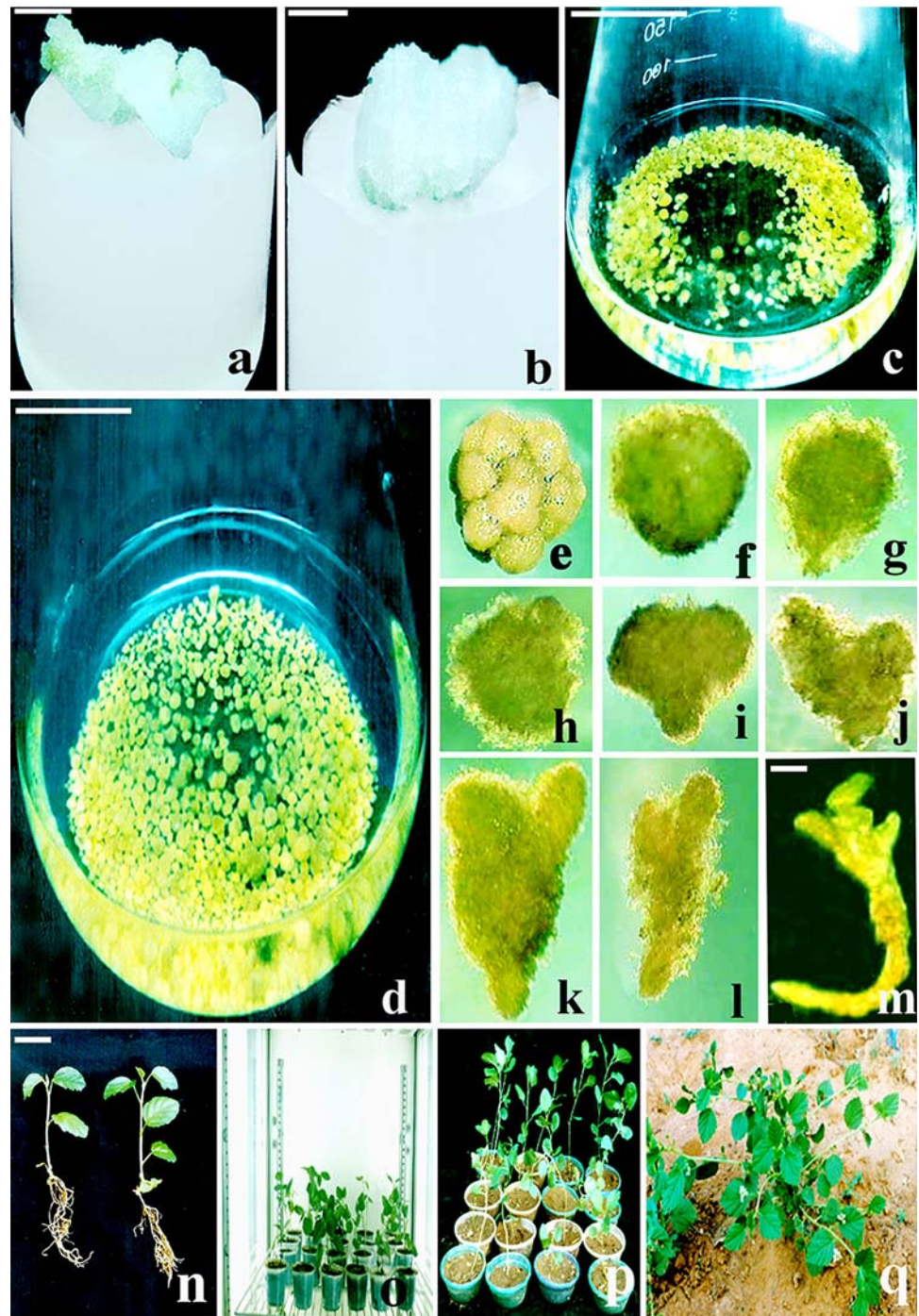
ITCLs explants. Similar observation was also put forth by Teixeira da Silva (2003).

Development of somatic embryos, germination and psoralen content

MS liquid medium supplemented with 1 μM NAA, 4 μM BA and different carbon sources were used to induce somatic embryos. The medium containing 30 g l<sup>-1</sup> sucrose

was more effective for promoting somatic embryogenesis than other concentrations of sucrose, glucose, fructose or maltose (Table 1). Similar results were also observed in *Cajanus cajan* and *Macrotyloma unifloram* by Patel et al. (1994) and Shamsudeen Varisai et al. (2004). Maltose was also ineffective for somatic embryogenesis in peanut (Eapen and George 1993). Somatic embryos number increased significantly from tTCLs embryogenic callus on MS medium containing 1 μM NAA, 4 μM BA and

**Fig. 3** Somatic embryogenesis and plant regeneration from TCLs hypocotyl explants of *P. corylifolia* L. **a** Whitish friable embryogenic callus from ITCL hypocotyl explants (*bar* 5 mm). **b** Whitish friable embryogenic callus from tTCL hypocotyl explants (*bar* 5 mm). **c** Different stages of somatic embryos on MS liquid medium + 1.0  $\mu$ M NAA + 4.0  $\mu$ M BA + 10.0  $\mu$ M glutamine + 2.0  $\mu$ M ABA (*bar* 10 mm). **d** Different stages of somatic embryos on MS liquid medium + 1.0  $\mu$ M NAA + 4.0  $\mu$ M BA + 15.0  $\mu$ M glutamine + 2.0  $\mu$ M ABA (*bar* 10 mm). **e–l** Different developmental stages of somatic embryos from suspension culture ( $\times 10$ ). **m** Germinated embryos (*bar* 5 mm). **n** Induction of roots on moist soil mixture and vermiculite (3:1) (*bar* 5 mm). **o** Acclimatized plants in growth chamber. **p** Regenerated plants in greenhouse. **q** Ex vitro plants of *P. corylifolia* after 3 months in the field



30 g l<sup>-1</sup> sucrose and different concentrations and combinations of glutamine and ABA. The media containing 15  $\mu$ M glutamine, and 3  $\mu$ M ABA or 10  $\mu$ M glutamine and 2  $\mu$ M ABA considerably increased the number of somatic embryos (Fig. 3c). Finer and Nagasawa (1988) and Lee et al. (2002) reported that glutamine and ABA were effective in somatic embryogenesis in *Glycine max* and *Aralia cordata*. ABA was also documented to regulate

normal development of somatic embryos in *Daucus carota* (Nishiwaki et al. 2000). In the present study, media containing 15  $\mu$ M glutamine and 2  $\mu$ M ABA produced the greatest number of embryos (Table 2; Fig. 3d) in different developmental stages (Fig. 3e–l). Increasing the concentrations of glutamine and ABA in the medium decreased the number of somatic embryos (Table 2). The germination of torpedo embryos was achieved in control medium

**Table 1** Influence of carbon source on somatic embryos number of rTCL hypocotyl explants of *Psoralea corylifolia* L. on MS liquid medium containing 1  $\mu\text{M}$  NAA and 4  $\mu\text{M}$  BA

Carbon source ( $\text{g l}^{-1}$ )	Number of somatic embryos per culture (mean $\pm$ SE)		
	Globular	Heart	Torpedo
<b>Glucose</b>			
10	7.4 $\pm$ 0.31 jk	3.6 $\pm$ 0.17 j	1.6 $\pm$ 0.24 hi
20	9.2 $\pm$ 0.36 i	4.8 $\pm$ 0.25 h	2.4 $\pm$ 0.21 g
30	12.6 $\pm$ 0.42 g	8.3 $\pm$ 0.31 ef	3.8 $\pm$ 0.14 ef
40	14.8 $\pm$ 0.34 f	6.4 $\pm$ 0.28 g	3.2 $\pm$ 0.17 f
50	8.2 $\pm$ 0.37 ij	4.2 $\pm$ 0.16 i	1.4 $\pm$ 0.12 i
<b>Fructose</b>			
10	9.4 $\pm$ 0.32 hi	4.6 $\pm$ 0.26 hi	2.3 $\pm$ 0.18 gh
20	16.8 $\pm$ 0.27 de	9.2 $\pm$ 0.34 e	3.8 $\pm$ 0.15 ef
30	10.6 $\pm$ 0.35 h	5.3 $\pm$ 0.38 gh	2.7 $\pm$ 0.19 fg
40	7.5 $\pm$ 0.28 j	3.7 $\pm$ 0.25 ij	1.8 $\pm$ 0.12 h
50	4.8 $\pm$ 0.17 l	2.4 $\pm$ 0.14 k	1.2 $\pm$ 0.06 ij
<b>Sucrose</b>			
10	18.4 $\pm$ 0.61 d	12.2 $\pm$ 0.38 d	8.4 $\pm$ 0.21 d
20	32.6 $\pm$ 0.72 c	26.0 $\pm$ 0.46 b	14.2 $\pm$ 0.26 c
30	47.6 $\pm$ 0.54 a	34.8 $\pm$ 0.42 a	22.4 $\pm$ 0.24 a
40	38.4 $\pm$ 0.65 b	24.2 $\pm$ 0.57 bc	16.0 $\pm$ 0.35 b
50	16.0 $\pm$ 0.52 e	8.2 $\pm$ 0.34 f	4.6 $\pm$ 0.28 e
<b>Maltose</b>			
10	4.6 $\pm$ 0.17 lm	2.6 $\pm$ 0.18 jk	1.4 $\pm$ 0.08 i
20	10.6 $\pm$ 0.14 h	5.3 $\pm$ 0.15 gh	2.7 $\pm$ 0.06 fg
30	6.5 $\pm$ 0.09 k	3.7 $\pm$ 0.09 ij	1.8 $\pm$ 0.05 h
40	2.4 $\pm$ 0.06 m	1.2 $\pm$ 0.07 kl	1.2 $\pm$ 0.02 ij
50	ND	ND	ND

The data were recorded after 4 weeks of culture

Values are mean of 50 explants per treatment and repeated thrice. Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by Duncan's multiple range test (DMRT)

ND not determined due to nil response

( $\frac{1}{2}$  MS + 20  $\text{g l}^{-1}$ ) as well as in different concentrations and combinations of BA, ABA and  $\text{GA}_3$ . However, the frequency of germination significantly increased in BA, ABA and  $\text{GA}_3$  combinations (Fig. 4). The maximum frequency of germination (97.6%) was obtained on medium containing 20  $\text{g l}^{-1}$  sucrose and 8  $\text{g l}^{-1}$  agar and supplemented with 2  $\mu\text{M}$  BA, 1  $\mu\text{M}$  ABA, 2  $\mu\text{M}$   $\text{GA}_3$  (Fig. 4). Cytokinin alone or in combination with  $\text{GA}_3$  or ABA developed embryo maturation and subsequent development of embryos into plantlets (Rout and Samantaray 1995). In the present study, higher concentration (2  $\mu\text{M}$ ) of ABA reduced the frequency of germination significantly. Attree and Fowke (1993) reported that ABA-enriched medium might prevent precocious germination and accumulation of high level of storage proteins. In our study, the somatic embryos produced green cotyledons, a plumule, and radicular zone without showing secondary callus (Fig. 3m), and subsequently developed into plantlets with shoot and root systems. The present investigation showed an enhancement of germination of somatic embryos within 2 weeks. Thus, we demonstrated that BA, ABA and  $\text{GA}_3$  combination are essential for higher germination of *P. corylifolia*.

#### Acclimatization

The plantlets generated further shoot and root systems in moist soil mixture and vermiculite (3:1 v/v) within 1 week. An average number of 4.0 roots per shoot with a 40–50 mm length were observed after 2 weeks of plantation in the water-irrigated control that was infected by fungal contamination. An average number of 16.5 roots per shoot with a 75–90 mm length were obtained in  $\frac{1}{2}$ -MS basal salt solution supplemented with 5  $\mu\text{M}$  IBA and 100  $\text{mg l}^{-1}$  BVN (Fig. 3n). The addition of BVN in the  $\frac{1}{8}$ -MS basal salt solution prevented fungal contamination of the moist soil mixture and favoured shoot growth. BVN is a systemic fungicide that belongs to benzimidazole family, which also showed a marked cytokinin like activity in *Bacopa monnieri* and *Psoralea corylifolia* (Tiwari et al. 2006; Baskaran and Jayabalan 2008). The plantlets were acclimatized in growth chamber (Fig. 3o). The plants were transferred to mixture of soil and organic manure (2:1, v/v) and maintained in the greenhouse (Fig. 3p). The regenerated plants were successfully established in the field (Fig. 3q). About 200 plants were tested for the survival rate in different

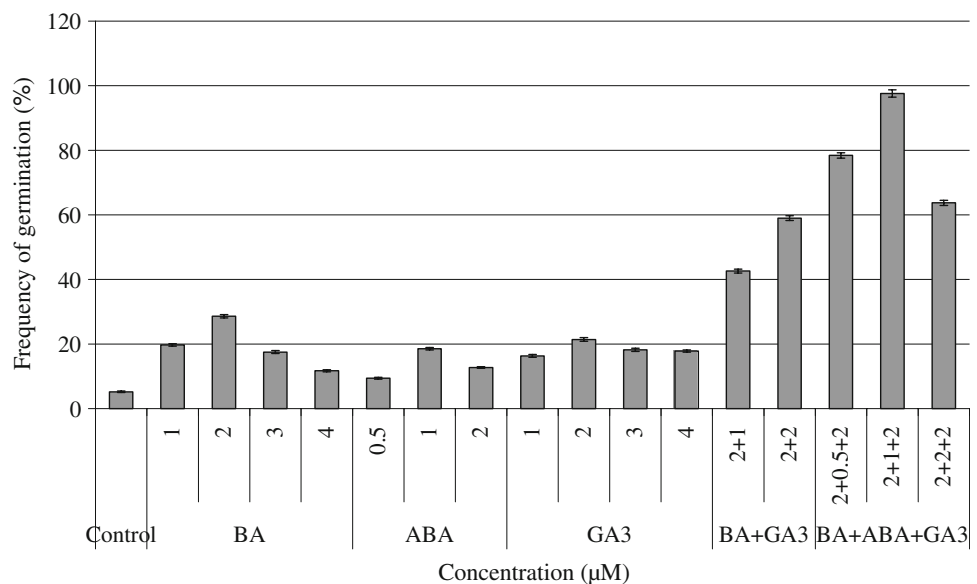
**Table 2** Influence of glutamine and ABA on somatic embryos number of rTCL hypocotyl explants of *Psoralea corylifolia* L. on MS liquid medium containing 1  $\mu\text{M}$  NAA, 4  $\mu\text{M}$  BA and 30  $\text{g l}^{-1}$  sucrose

Growth substances ( $\mu\text{M}$ )		Number of somatic embryos per culture (mean $\pm$ SE)		
		Globular	Heart	Torpedo
Glutamine				
5		27.6 $\pm$ 0.42 k	14.0 $\pm$ 0.27 m	8.4 $\pm$ 0.18 l
10		35.7 $\pm$ 0.59 ij	27.4 $\pm$ 0.31 hi	16.5 $\pm$ 0.24 h
15		46.0 $\pm$ 0.45 fg	34.6 $\pm$ 0.36 fg	25.4 $\pm$ 0.21 e
20		32.2 $\pm$ 0.56 j	20.5 $\pm$ 0.42 jk	14.7 $\pm$ 0.32 i
25		20.4 $\pm$ 0.47 mn	11.6 $\pm$ 0.23 n	6.8 $\pm$ 0.26 lm
ABA				
1		30.2 $\pm$ 0.37 jk	16.8 $\pm$ 0.25 l	9.2 $\pm$ 0.24 kl
2		37.4 $\pm$ 0.46 hi	29.6 $\pm$ 0.28 h	18.4 $\pm$ 0.18 gh
3		48.6 $\pm$ 0.32 f	36.0 $\pm$ 0.32 ef	28.0 $\pm$ 0.26 d
4		36.0 $\pm$ 0.54 i	24.2 $\pm$ 0.36 ij	16.2 $\pm$ 0.15 hi
5		23.8 $\pm$ 0.38 m	11.7 $\pm$ 0.28 mn	5.3 $\pm$ 0.12 m
Glutamine	ABA			
10	1	54.6 $\pm$ 1.26 e	38.2 $\pm$ 0.57 e	24.8 $\pm$ 0.42 ef
10	2	62.4 $\pm$ 1.18 c	49.4 $\pm$ 0.46 b	37.6 $\pm$ 0.37 b
10	3	46.0 $\pm$ 0.84 fg	23.0 $\pm$ 0.35 j	16.5 $\pm$ 0.28 h
15	1	84.5 $\pm$ 1.42 b	64.2 $\pm$ 0.92 c	46.2 $\pm$ 0.31 c
15	2	123.7 $\pm$ 1.24 a	108.0 $\pm$ 0.86 a	93.2 $\pm$ 0.25 a
15	3	59.4 $\pm$ 1.06 cd	43.6 $\pm$ 0.74 cd	27.6 $\pm$ 0.28 de
20	1	38.0 $\pm$ 0.67 h	24.6 $\pm$ 0.46 i	12.3 $\pm$ 0.36 j
20	2	42.6 $\pm$ 0.56 g	34.8 $\pm$ 0.42 f	19.4 $\pm$ 0.26 g
20	3	27.4 $\pm$ 0.42 kl	14.2 $\pm$ 0.28 lm	9.6 $\pm$ 0.15 k

The data were recorded after 4 weeks of culture

Values are mean of 50 explants per treatment and repeated thrice. Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by Duncan's multiple range test (DMRT)

**Fig. 4** Influence of BA, ABA and GA<sub>3</sub> in  $\frac{1}{2}$  MS medium with 20  $\text{g l}^{-1}$  sucrose on the germination of somatic embryos of *P. corylifolia*. The results are expressed as the means with SE from three replicates per treatment. The data were recorded after 2 weeks of transfer of somatic embryos to media with the indicated plant growth regulator composition



seasons. The survival rate during summer was 55–60% whereas in winter it was 100%. High temperatures (36–45°C) could be unfavourable for the establishment of plantlets in the field, whereas relatively low temperatures

(25–28°C) during winter could be favourable for establishment. The study therefore suggests that for *P. corylifolia* hardened plants should be transferred to field only during winter season for the best survival rate.

## Psoralen content

Somatic embryos were used to test production of psoralen content. A psoralen content of  $12.9 \mu\text{g g}^{-1}$  DW was detected in embryos. Supporting this observation, eleutheroside content was investigated by Jeong et al. (2005) in in vitro regenerated embryos of *Eleutherococcus chiisanensis*. The tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions. Several products were found to be accumulating in cultured cells at a higher level through optimization of cultural conditions (Mulabagal and Tsay 2004). The present study revealed the psoralen content in different developmental stages of embryos of *P. corylifolia*. This study should facilitate improvement of psoralen production in future studies.

In the present study, we have provided a protocol for the high frequency of somatic embryogenesis of *P. corylifolia* from hypocotyl tTCLs explants through cell suspension culture. Higher frequency of somatic embryogenesis was dependent on TCL system, concentration and combination of growth regulators, especially glutamine and ABA. This protocol can be helpful for conservation strategy, phyto-medicine production, somaclonal variation and genetic transformation studies. To our knowledge, this is the first report of the successful somatic embryogenesis via suspension culture in *P. corylifolia*.

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