Continuous somatic embryogenesis and plant regeneration from hypocotyl segments of *Psoralea corylifolia* Linn., an endangered and medicinally important Fabaceae plant

Ashok Kumar Sahrawat* and Suresh Chand

Plant Tissue Culture and Genetics Research Group, School of Life Sciences, Devi Ahilya University, Khandwa Road Campus, Indore 452 017, India

An efficient *in vitro* plant regeneration system has been developed for rapid and continuous production of somatic embryos from hypocotyl segments of *Psoralea corylifolia* Linn., an endangered and medicinally important plant species belonging to family Fabaceae. Embryogenic callus was induced from hypocotyl segments on Murashige and Skoog medium supplemented with 2.7–10.8 µM α-naphthaleneacetic acid (NAA) and 2.2 µM 6-benzylaminopurine (BAP). High-frequency somatic embryogenesis was achieved after transfer of embryogenic callus clumps to MS medium supplemented with 1.4 µM NAA and 2.2 µM BAP, alone or in combination with 0.9–2.8 µM abscisic acid (ABA). The addition of 0.6 or 1.2 mM L-glutamine to the MS medium containing 2.7 µM NAA, 2.2 µM BAP, and 0.9 µM ABA significantly enhanced maturation of somatic embryos to cotyledonary-stage. The maximum number of cotyledonary-stage embryos (48.3 ± 0.87) was obtained after 16 weeks of culture on MS medium containing 2.7 µM NAA, 2.2 µM BAP, 0.94 µM ABA and 1.2 mM L-glutamine. Well-developed embryos germinated on ½ MS0, MS0 (MS medium without any growth regulator) and also on MS medium supplemented with BAP (2.2–8.8 µM). Somatic embryo-derived plants were transferred to pots, where they grew well and attained maturity. The system of somatic embryogenesis described here will facilitate tissue culture, germplasm conservation and gene transfer research on *P. corylifolia*.

PSORALEA corylifolia Linn., commonly known as Babchi, is an endangered and medicinally important plant belonging to the family Fabaceae. The plant is well recognized in Chinese and Indian folkloric medicine as a laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic in febrile conditions. The seeds have been recommended in the treatment of leucoderma, leprosy, psoriasis and inflammatory diseases of the skin[1]. Recently, phenolic compounds of *P. corylifolia* have been reported to be effective in protecting cellular constituents and functions against oxidative stresses[2]. The exploitation of this medicinally important plant from the wild and natural habitat and inadequate efforts for cultivation have resulted in a marked decline in the population of this species. *P. corylifolia* is propagated by seed germination. A conventional method of propagation of *P. corylifolia* through seeds is not adequate, as seeds need pretreatment because of hard seed coat[3]. Hence, it is felt that there is a great need for cultivation of this medicinally important plant.

Regeneration of plants *in vitro* via somatic embryogenesis has some distinct features such as single-cell origin, the consequent low frequency of chimeras and the production of a high number of regenerates[4,5]. Somatic embryogenesis has several other advantages, including the efficiency of process (i.e. the formation of plantlets in fewer steps, with a concomitant reduction in labour, time and cost) and the morphological and cytological uniformity of the plantlets[6]. Somatic embryogenesis has been achieved in many legume species[7–13], but to date, there is no report on somatic embryogenesis in *P. corylifolia*. Availability of an efficient plant regeneration system via continuous production of somatic embryos of this plant will be useful in micropropagation, germplasm conservation and genetic transformation studies.

We report continuous somatic embryogenesis and plant regeneration from hypocotyl segments of *P. corylifolia* Linn., a plant of considerable importance as source of medicinal compounds.

Seeds of *P. corylifolia* Linn. were obtained from the Jawaharlal Nehru Agricultural University Campus, Indore. Seeds were surface-disinfected with freshly prepared 0.1% (w/v) aqueous mercuric chloride solution for 10 min, followed by a quick rinse (10 s) in 70% (v/v) ethanol and 3–4 washings in sterile distilled water. An average of 5–7 seeds were aseptically cultured on 25 mm × 150 mm glass tubes (Borosil, Mumbai) containing 25 ml of the medium consisting of Murashige and Skoog[14] medium and 3.0% sucrose (w/v) without any growth regulators. The culture tubes were capped with non-absorbent cotton plugs wrapped with one layer of cheesecloth. The medium was solidified with 0.8% (w/v) agar (Hi-Media, India) and autoclaved at 121°C and 1.06 kg/cm^2 pressure for 15 min, after adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N NaOH. The cultures were incubated under 16/8-h light/dark photoperiod (cool-white fluorescent light, irradiance 50 µmol m^{-2} s^{-1}) at 25 ± 2°C and a relative humidity of 60%. Hypocotyl segments (0.5 cm) collected from 7-day-old *in vitro*-grown seedlings were used as explants.

For callus induction, hypocotyl segments were cultured on MS medium supplemented with different concentrations of α-naphthaleneacetic acid (NAA, 2.7–10.8 µM) in combination with 6-benzylaminopurine (BAP, 2.2 µM) and sucrose (3.0%, w/v). Each treatment consisted of seven replicates and was repeated thrice. Explants forming embryogenic callus were scored eight weeks after culture initiation and percentage of response was calculated.

*For correspondence. (e-mail: ashoksahrawat@hotmail.com)

For the induction and development of somatic embryos, embryogenic calli were transferred to various media, MS + 1.4 μM NAA + 2.2 μM BAP; MS + 1.4 μM NAA + 2.2 μM BAP + 0.9; 1.8; or 2.8 μM abscisic acid (ABA); MS + 1.4 μM NAA + 2.2 μM BAP + 0.9 μM ABA + 0.6 or 1.2 mM L-glutamine. Each treatment consisted of seven replicates per treatment and was repeated thrice. The average number of cotyledonary-stage somatic embryos was scored after 8, 16, 32, 48 and 64 weeks. For germination, cotyledonary-stage somatic embryos were transferred to ½ MS0, MS0 or MS medium containing 2.2, 4.4, 6.6 or 8.8 μM BAP. The experiment was repeated thrice, with ten replicates per treatment. The frequency of germination of somatic embryos was scored four weeks after their transfer to the germination medium.

The cultures were transferred to the fresh medium after an interval of 4 weeks. For long-term maintenance and embryogenesis, cultures were maintained on MS medium supplemented with 1.4 μM NAA, 2.2 μM BAP, 0.9 μM ABA and 1.2 mM L-glutamine and for germination of somatic embryos into plants, MS medium enriched with 6.6 μM BAP was used. Regenerated plants with well-developed root systems were initially maintained on liquid half-strength MS medium without growth regulators, for hardening. After a week, the rooted plants were transferred to pots containing an autoclaved soil : manure : peat moss : sand (1 : 2 : 2 : 1) mixture.

Statistical analysis was carried out using Fisher's least significant difference (LSD) test. Standard error of the mean was calculated. $P < 0.05$ was considered significant.

Callus was developed first at the cut ends of hypocotyl segments within three weeks, when cultured on MS medium supplemented with different concentrations of NAA (2.7–10.8 μM) in combination with BAP (2.2 μM). Embryogenic callus developed all over the surface of hypocotyl segments within eight weeks of culture initiation. The frequency of embryogenic callus induction ranged from 47.6 to 85.7% on MS medium containing different concentrations of NAA. The highest frequency (85.7%) was observed on the medium containing 8.1 μM NAA. Calli became more organized and nodular after transfer to a medium supplemented with 1.4 μM NAA, 2.2 μM BAP and 0.9–2.8 μM ABA. Globular somatic embryos appeared on the surface of the calli within four weeks (Figure 1 *a*). During subculture, these globular embryos further developed into cotyledonary-stage embryos and attained maturity on the same medium (Figure 1 *b*). It has been reported that on proliferation medium, callus differentiates localized groups of meristematic cells called proembryogenic masses[15]. In repeated subcultures, the embryogenic cells continue to multiply without the appearance of embryos. However, they develop into embryos after transfer to embryo development medium containing a low level of auxin. Halperin and Wetherell[16] first recognized the importance of auxin for somatic embryogenesis. Since then, several studies have shown that the process of embryogenesis takes place with the induction of cells with embryogenic competence in the presence of high concentration of auxin, and the development of embryogenic competence cells into embryos takes place in the absence of or in the presence of low concentration of auxin[15,17]. It has been reported that somatic embryogenesis in cultures proceeds from cells, which are already committed for embryogenic development[6]. This requires growth regulators and favourable conditions to allow these pre-determined cells to undergo cell division and expression of embryogenesis[18].

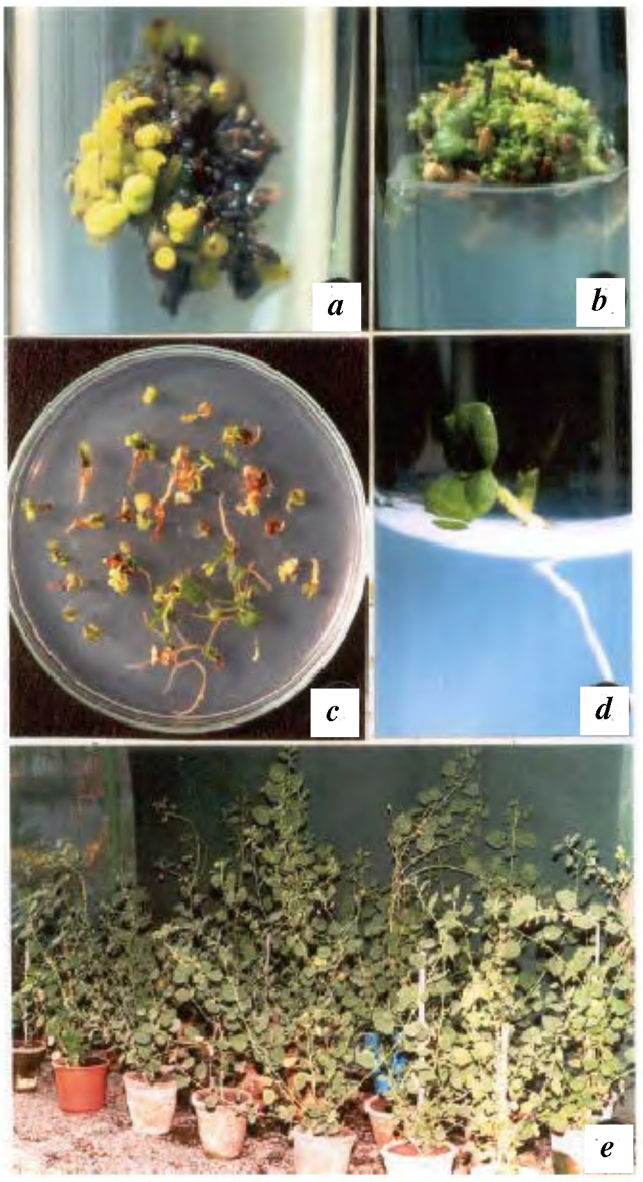

Figure 1. Somatic embryogenesis from embryogenic calli derived from hypocotyl segments of *Psoralea corylifolia* Linn. ***a***, Somatic embryo development on MS medium supplemented with 2.7 μM NAA, 2.2 μM BAP and 0.9 μM ABA; ***b***, Cotyledonary-stage embryos on MS medium containing 2.7 μM NAA, 2.2 μM BAP and 0.9 μM ABA; ***c***, Various stages of somatic embryos germination as observed on MS medium supplemented with 6.6 μM BAP; ***d***, Well-germinated somatic embryo showing root and cotyledonary leaves; and ***e***, Somatic embryo-derived plants in pots in the greenhouse.

It was observed that different media varied in their effectiveness for the development and maturation of somatic embryos to cotyledonary-stage. Table 1 shows the mean number of cotyledonary-stage somatic embryos after 8, 16, 32, 48 and 64 weeks. The maximum number of cotyledonary-stage embryos was recorded 16 weeks after transfer of callus clumps to MS medium containing 1.4 µM NAA, 2.2 µM BAP, 0.9 µM ABA and 1.2 mM L-glutamine. The addition of ABA (0.9–2.8 µM) into the medium resulted in enhanced production of cotyledonary-stage embryos over a period of 8, 16, 32, 48 and 64 weeks. The frequency of cotyledonary stage somatic embryos was significantly ($P < 0.05$) enhanced when 0.6 or 1.2 mM L-glutamine was added to the medium supplemented with 1.4 µM NAA, 2.2 µM BAP and 0.9 µM ABA. Enhanced production of cotyledonary-stage embryos on ABA-enriched media might be due to its ability to prevent precocious germination and promote normal development of embryos by suppression of secondary embryogenesis[4]. In embryogenic cultures of caraway, ABA inhibited precocious germination of embryos and promoted a normal course of ontogeny and maturation of embryos[19]. Fujii *et al.*[20] reported embryo maturation with ABA in alfalfa. The effect was due to accumulation of embryo-specific reserves such as carbohydrates. Roberts *et al.*[21] reported that somatic embryos of interior spruce required 40–60 µM ABA to completely stop precocious germination and promote the formation of mature embryos. The nitrogen content of the culture medium has been reported to influence the morphogenic effects of growth substances[22]. L-glutamine has been reported critical for embryogenesis among the amino acids used as a source of nitrogen[22–24]. In orchardgrass, embryos formed on amino acid-containing medium showed high percentage of conversion and considerably less incidence of precocious germination[25]. The yield of alfalfa somatic embryos was also considerably improved when amino acids such as proline, alanine, arginine and glutamine were added to the callus-maintenance medium[26]. There are reports to suggest that changes in gene expression occur when the somatic cells embark on embryogenic development, by the synthesis of embryo-specific proteins and increased enzyme activities in the pyrimidine pathway[27,28]. Several molecular markers indicating the transition of somatic cells into embryogenic cells have been recognized, and more than 21 embryo-specific genes have been cloned from somatic embryos[29].

Table 1 shows mean number of cotyledonary-stage embryos after 8, 16, 32 and 64 weeks. The maximum response for cotyledonary-stage embryos was observed after 16 weeks of culture. The decline in cotyledonary-stage embryos after 32 weeks may be due to the altered hormonal balance within the cells and/or sensitivity of the cells to exogenous growth substances. With an increase in culture duration, Molle *et al.*[30] observed progressive loss of embryogenic potential, decline in embryo quality and variation in growth in carrot cultures. Possibly, in *P. corylifolia*, the decline in cotyledonary-stage embryos may be attributed to the endogenous level of growth hormone and sensitivity to the growth regulators, specially auxin and cytokinin supplemented to the culture medium. After embryo induction, the role of auxin changes in that the embryos start to synthesize their own auxin[31]. Several studies have shown that proper polar transport of auxin is a pre-requisite for normal embryogenesis beyond the globular stage[32,33].

For germination, cotyledonary-stage somatic embryos were individually separated and cultured on half-strength MS medium or MS medium without any growth regulator. Germination of somatic embryos started within two weeks after their transfer to the germination medium. The frequency of somatic embryo germination was 23.3% on ½ MS0 and 36.6 % on MS0 medium. Incorporation of BAP (2.2–8.8 µM) into the MS medium significantly ($P < 0.05$) increased germination frequency of somatic embryos (Table 2). The highest frequency (80.0%) of somatic embryo germination was observed on MS medium supplemented with 6.6 µM BAP. Vlasinova and Havel[34] reported continuous somatic embryogenesis in Japanese maple on MS medium supplemented with

Table 1. Mean number of cotyledonary-stage embryos from hypocotyl segment-derived calli of *Psoralea corylifolia* Linn. maintained on various media over a period of 64 weeks. Data represent mean ± standard error of three experiments, each containing 7 replicates. Fisher's least significant difference (LSD) was used to compare means

Medium	Cotyledonary-stage embryo				
	8 weeks	16 weeks	32 weeks	48 weeks	64 weeks
MS + 1.4 µM NAA + 2.2 µM BAP	21.2 ± 0.61	23.4 ± 0.83	14.3 ± 0.59	9.5 ± 0.30	3.1 ± 0.38
MS + 1.4 µM NAA + 2.2 µM BAP + 0.9 µM ABA	30.4 ± 0.94	34.8 ± 1.38	18.4 ± 0.95	12.3 ± 0.60	6.4 ± 0.29
MS + 1.4 µM NAA + 2.2 µM BAP + 1.9 µM ABA	37.9 ± 1.0	42.5 ± 1.0	28.5 ± 0.73	18.4 ± 0.73	10.6 ± 0.39
MS + 1.4 µM NAA + 2.2 µM BAP + 2.8 µM ABA	34.8 ± 0.83	36.5 ± 0.25	24.7 ± 0.59	12.5 ± 0.31	7.1 ± 0.55
MS + 1.4 µM NAA + 2.2 µM BAP + 0.9 µM ABA + 0.6 mM L-glutamine	40.2 ± 1.1	44.7 ± 1.15	23.8 ± 0.82	17.5 ± 0.58	6.1 ± 0.36
MS + 1.4 µM NAA + 2.2 µM BAP + 0.9 µM ABA + 1.2 mM L-glutamine	43.7 ± 0.87	48.3 ± 0.87	35.8 ± 1.3	25.6 ± 0.67	14.3 ± 0.28
LSD at 5% level	1.66	3.66	2.70	3.50	1.56

Table 2. Effect of BAP on germination of somatic embryo. Data represents mean ± standard error of three experiments, each consisting of 10 somatic embryos. Fisher's least significant difference (LSD) was used to compare means

Medium + growth regulator (μM)	Germination frequency (mean ± SE)
1/2 MS0	23.3 ± 0.56
MS0	36.6 ± 0.66
MS + BAP (2.2)	46.6 ± 1.21
MS + BAP (4.4)	63.7 ± 0.88
MS + BAP (6.6)	80.0 ± 1.80
MS + BAP (8.8)	66.7 ± 0.66
LSD at 5 % level	20.11

0.05 μM BAP and 0.5 μM NAA. In our study, cultures derived from hypocotyl segments of *P. corylifolia* are able to produce somatic embryos continuously on MS medium containing 1.4 μM NAA, 2.2 μM BAP, 0.9 μM ABA and 1.2 mM L-glutamine. The germination of somatic embryos into plantlets continued even after two years of culture on MS medium supplemented with 6.6 μM BAP. Venkatachalam *et al.*[11] and Chengalrayan *et al.*[35] reported the development of plantlets from embryos of groundnut after exposure to BAP. Germination of somatic embryos was characterized by simultaneous production of shoot and root (Figure 1 *c*, *d*). Germinated embryos produced good root and shoot systems when transferred to growth regulator-free half-strength MS liquid medium. They developed into complete plantlets within a week. Plantlets were transferred to pots where they grew well, attained maturity and set viable seeds (Figure 1 *e*). Organized development leading to plantlet regeneration via embryogenesis has successfully been achieved in many plant species through judicious selection of the inoculum, proper choice of the medium, including the growth-active substances, and the control of the physical environment[5,36,37].

We have developed an expeditious protocol for large-scale production of plants via continuous somatic embryogenesis in *P. corylifolia*. The plants developed by the protocol were normal, healthy and produced viable seeds.

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