Embryogenesis and plant regeneration from unpollinated ovary culture of *Psoralea corylifolia*

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Abstract

Embryogenesis and plant regeneration was achieved from callus cultures derived from unpollinated ovaries of *Psoralea corylifolia* L. Callus was initiated from unpollinated ovaries on Murashige and Skoog (MS) medium supplemented with 2.2 μM N^6-benzyladenine (BA) and various concentrations of α-naphthaleneacetic acid (NAA (2.7 to 10.7 μM) or 2,4-dichlorophenoxyacetic acid (2,4-D (2.3 to 9 μM) alone or in combination. Highly organized embryogenic callus induction, embryo development, proliferation and maturation were achieved on transfer of callus clumps to MS medium supplemented with NAA (0.27 μM) or 2,4-D (0.23 μM) alone or in combination with BA (2.2 to 8.8 μM). Addition of abscisic acid (ABA) (0.95 to 5.8 μM) to the medium enhanced average numbers of cotyledonary stage embryos, the maximum number (34.6 ± 0.7) being obtained on MS medium containing 0.27 μM NAA, 2.2 μM BA and 3.8 μM ABA. Embryos germinated on MS medium supplemented with BA (0 to 8.8 μM). MS medium containing gibberellic acid (GA_3 (0.29 to 5.8 μM) enhanced embryo germination frequency, the highest frequency (66.7 %) occurring on MS medium containing 2.2 μM BA and 4.3 μM GA_3. Effect of several concentrations (3.0 to 6.0 %) of sucrose or maltose was also observed on germination of embryos. MS medium enriched with maltose supported high frequency of embryo germination.

Additional key word: abscisic acid, auxins, cytokinins, gibberellic acid, gynogenic haploid, maltose, sucrose.

Introduction

Psoralea corylifolia L. is a rare, endangered and an important medicinal plant distributed in tropical and subtropical regions of the world. This plant is valuable for its seeds and vegetative parts, which are rich sources of numerous compounds such as furanocoumarines, coumesterol, chalcones and flavons (Satyavati *et al*. 1987).

Importance of haploids to produce homozygous diploids and selection of recombinants has been reported (Bajaj 1990). Currently, anther culture is the most successful method of producing haploids in many plant species (Hu and Yang 1986), while unpollinated ovaries or ovules culture has not been still fully exploited for crop improvement (Gu and Cheng 1983, Lakshmi Sita 1997). Gynogenesis can be very useful for the production of haploid plants in species where anther culture has given inadequate results (San and Gelebart 1986, Cappadocia and Vieth 1990). Ever since the first successful induction of *in vitro* gynogenetic haploid plants in barley (San Noeum 1976), efforts have been made for *in vitro* production of gynogenic haploid plants in several species belonging to the following families: *Poaceae*, *Asteraceae*, *Liliaceae*, *Solanaceae*, *Compositae*, *Moraceae*, *Scrophulariaceae*, *Salicaceae*, and *Euphorbiaceae* (Yang and Zhou 1990, Lakshmi Sita 1997, Chand and Basu 1998, Sibi *et al*. 2001, Alan *et al*. 2003). However, success with *in vitro* culture of unpollinated ovaries and ovules remains low due to problems in handling of juvenile tissues and isolating egg without injury (Rangan 1982, Yang and Zhou 1982, Lakshmi Sita 1997). Among all the factors affecting gynogenesis during culture, plant growth regulators,

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Abbreviations: ABA - abscisic acid; BA - N^6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; NAA - α-naphthaleneacetic acid; MS - Murashige and Skoog (1962); MSO - MS medium without any growth regulator.

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especially exogenous auxins, are known to play a crucial role in ovary culture because of the direct effect on callus induction as well as switching the pathway towards organogenesis and embryogenesis (Bajaj 1990, Castillo and Cistué 1993, Chand and Basu 1998, Alan *et al*. 2003, Sauer and Wilhelm 2005)). Depending upon the regenerative pathways, *in vitro* regenerated gynogenic plants differ in their ploidy level (Yang and Zhou 1990). Reports on *in vitro* gynogenesis have shown that gynogenic plants may be haploid, diploid or mixoploid (San and Gelebart 1986, Yang and Zhou 1990, Chand and Basu 1998, Alan *et al*. 2003), but show lower yield of albinism than the androgenetic one (San and Gelebart 1986, San Noeum 1976).

We report for the first time an efficient system for the production of haploid plants from unfertilized ovaries of *Psoralea corylifolia* L. The technique can be employed not only for increasing the medicinal compounds by studying the plants with different ploidy levels, but also preserving this endangered and valuable medicinally important plant species.

Materials and methods

Plants: Young flower buds (5 - 8 mm) of *Psoralea corylifolia* L. were obtained from plants grown at experimental farm of Jawaharlal Nehru Agricultural University, Campus Indore, India. Flower buds were collected as soon as the enveloping foliage leaves had exposed the buds. After cleaning with sterile distilled water, flower buds were surface sterilized with freshly prepared 0.1 % (m/v) aqueous mercuric chloride solution for 10 min with intermittent shaking followed by a quick rinse in 70 % (v/v) ethanol and then washed thoroughly 3 to 4 times in sterile distilled water.

Culture initiation and embryogenesis: Young unpollinated ovaries were dissected out from young flower buds of *P. corylifolia* and cultured in 25 × 150 mm glass tubes (*Borosil*, Bombay, India) containing 25 cm^3 of Murashige and Skoog (1962) medium supplemented with various concentrations of NAA (2.7, 5.4, 10.7 μM) or 2,4-D (2.3, 4.5, 9 μM) and BA (2.2 μM). In a separate experiment, effect of NAA (10.7 μM) and 2,4-D (2.3, 4.5, 9.0 μM) on callus induction was also evaluated. Culture tubes were capped with non-absorbent cotton plugs wrapped in one layer of cheesecloth. The pH of the medium was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH before adding 0.8 % (m/v) agar (Hi*Media*, Bombay, India) and autoclaved at 121 °C and 104 kPa for 15 min. The cultures were incubated under 16-h photoperiod (cool-white fluorescent light, irradiance of 50 μmol m^{-2} s^{-1}) at temperature of 25 ± 2 °C and relative humidity of 60 %. Each treatment consisted of seven replicates and was repeated three times. Explants forming embryogenic callus were scored eight weeks after culture initiation and percentage response was calculated.

For further proliferation and development of embryos, embryogenic calli were transferred to MS medium containing either NAA (0.27 μM) or 2,4-D (0.23 μM) alone or in combination with BA (2.2, 4.4, 6.6, 8.8 μM) or ABA (0.95, 1.9, 3.8, 5.8 μM). Each treatment consisted of five replicates and was repeated three times. Average numbers of cotyledonary stage embryos were scored eight weeks after culture.

Embryos germination: For germination, cotyledonary-stage embryos were transferred to various media, ½ MS, MS alone or with BAP (2.2, 4.4, 8.8 μM); MS supplemented with 2.2 μM BA and 0.29, 1.4, 2.9, 4.3, 5.8 μM GA_3; ½ MS supplemented with 4.4 μM BA alone or in combination with 4.3 μM GA_3. To study the effect of sugars embryos were cultured on MS medium containing 4.4 μM BA and 3.0 to 6.0 % sucrose or maltose. Each treatment consisted of five replicates and was repeated three times. Embryo germination frequency was scored four weeks after culture. Regenerated plantlets with well-developed root systems were initially maintained on liquid half-strength MS medium without growth regulators for hardening of roots. After a week, the rooted plantlets were transferred to pots containing autoclaved soil mixture, soil : manure : peat moss : sand at the ratio of 1:2:2:1.

Cytology of the regenerated plants: For cytological studies of chromosome numbers, root tips of the regenerated plants were washed with running tap water to remove all traces of culture medium, and fixed in Carnoy's fixative (ethyl alcohol:acetic acid mixture 3:1 v/v) for overnight followed by 2 % aceto-orcein: 1 M HCl mixture (9:1) for 1 h. Root tips were squashed in 45 % acetic acid and examined under the microscope.

Statistical analysis: For each treatment, data pertaining to embryogenic callus induction frequency, average number of cotyledonary embryos and germination frequency of embryos were statistically analyzed using Student's *t*-test.

Results and discussion

Callus induction and embryogenesis: Swelling in the unpollinated ovaries was observed 11 d after culture on all the media tested and callus formation started at the base of the ovaries (Fig. 1*A*). Callus proliferated on the

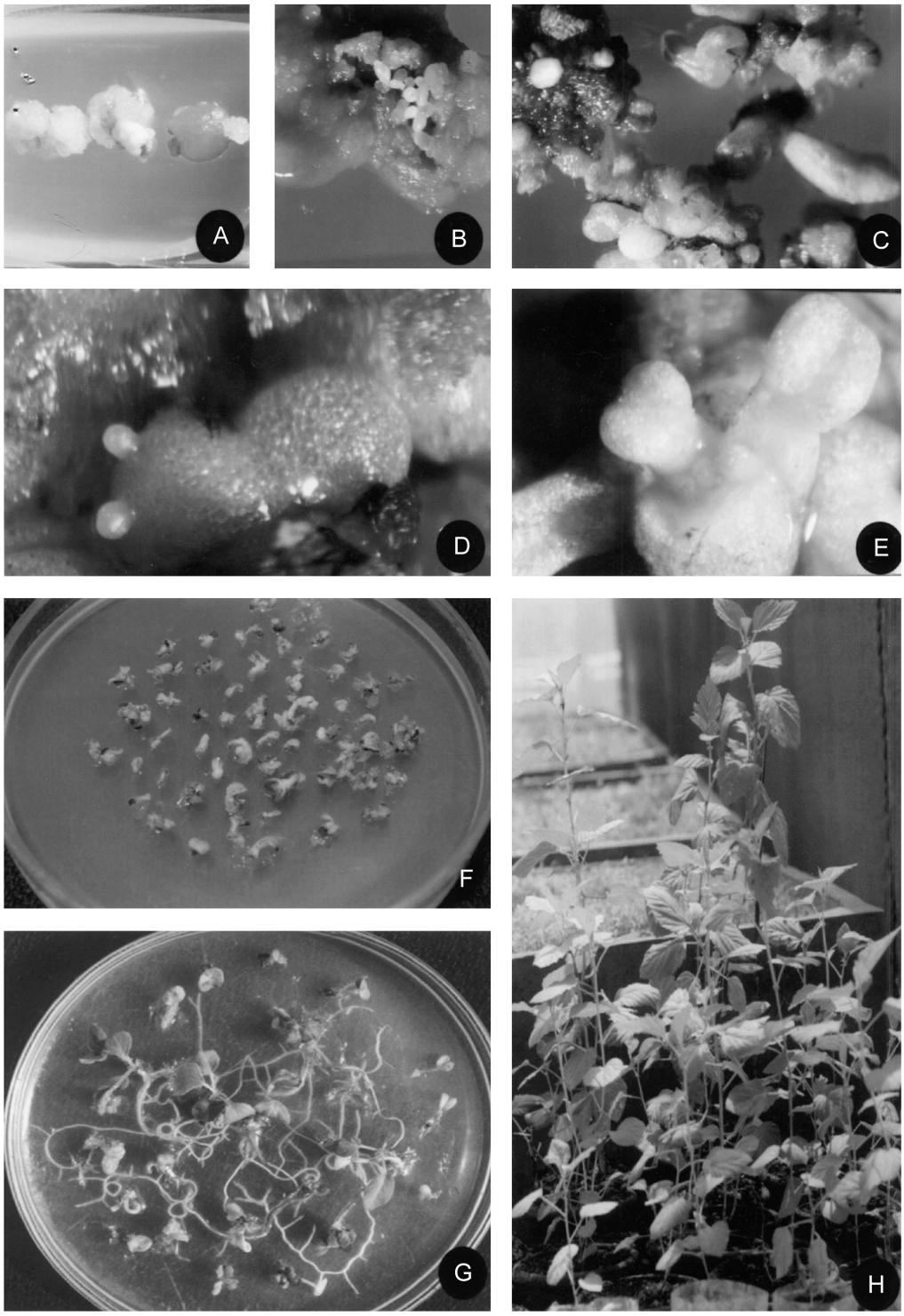

Fig. 1. Embryogenic callus induction and plant regeneration from unpollinated ovaries of *Psoralea corylifolia* Linn. *A* - initiation of callus from unpollinated ovaries after three weeks of culture on MS medium containing 10.7 µM NAA, 4.5 µM 2,4-D and 2.2 µM BA, *B* - globular embryos after 8 weeks of culture on MS medium containing 10.7 µM NAA, 4.5 µM 2,4-D and 2.2 µM BA, *C* - various stages of embryos (globular, torpedo and cotyledonary stage) on MS medium containing 0.27 µM NAA and 6.6 µM BA, *D,E* - initiation and development of secondary embryos on MS medium supplemented with 0.23 µM 2,4-D and 2.2 µM BA, *F,G* - germination of embryos on MS medium supplemented with 2.2 µM BA and 4.3 µM GA_3, *H* - regenerated plants transferred to pots.

Table 1. Callus induction frequency from unpollinated ovaries of *Psoralea corylifolia* L. on MS medium supplemented with different concentrations of 2,4-D or NAA in combination with 2.2 μM BA. Evaluation 8 weeks after culture initiation. Experiment contained seven replicates per treatment and was repeated thrice. Means ± SE. * - significant difference between NAA + 2,4-D and 2,4-D or NAA alone.

NAA [μM]	2,4-D [μM]	Callus induction [%]
2.7	-	16.7 ± 0.55
5.4	-	23.8 ± 0.67
10.7	-	28.5 ± 0.57
-	2.3	9.5 ± 0.33
-	4.5	14.2 ± 0.55
-	9.0	19.0 ± 0.88
10.7	2.3	38.2 ± 0.82*
10.7	4.5	42.8 ± 0.65*
10.7	9.0	35.8 ± 0.78*

same medium. Percent response for callus formation on the various media varied and maximum response (42.8 %) was observed on MS medium containing 10.7 μM NAA, 4.5 μM 2,4-D and 2.2 μM BA (Table 1). Whereas, MS medium containing 2.3 μM 2,4-D and 2.2 μM BA was the least effective (9.5 %). MS medium supplemented with NAA showed better response for callus induction than the medium containing 2,4-D. Interestingly, the presence of both NAA and 2,4-D into medium enhanced ($P < 0.05$ to 0.1) callus induction frequency. The high frequency of callus induction from ovaries might be due to a synergistic effect of these two auxins. Castillo and Cistué (1993) reported high frequency of callus induction, from unpollinated ovaries of barley placed on a callus induction medium containing two auxins, MCPA (4-chloro-2-methylphenoxyacetic acid) and IAA, and a cytokinin, BA. Auxins are known to trigger the stimulation of pre-embryogenic determined cells to undergo cell division and then expression of embryogenesis (Fehér *et al*. 2003). Within eight weeks of culture, globular embryos appeared on the surface of calli (Fig. 1*B*). These embryos further developed and proliferated on MS medium containing low concentration of NAA (0.27 μM) or 2,4-D (0.23 μM) alone or in combination with BA (2.2 to 8.8 μM). On this medium, various stages of well-developed embryos (globular to cotyledonary stage) were observed (Fig. 1*C*). Induction and development of secondary embryos was also observed on MS medium containing 0.23 μM 2,4-D and 2.2 μM BA (Fig. 1*D*,*E*). Depending on the media, maturation of embryos varied (Table 2). Transition of globular stage embryos to cotyledonary stage embryos enhanced significantly ($P < 0.05$ to 0.01) with the addition of BA (2.2 to 8.8 μM) into MS medium containing either NAA (2.7 μM) or 2,4-D (2.3 μM). Furthermore, the conversion of globular stage embryos to cotyledonary stage embryos was increased significantly ($P < 0.001$) with the addition of ABA (0.95 to 5.8 μM) into MS medium containing 0.27 μM NAA and 2.2 μM BA, with maximum numbers (34.6 ± 0.7) on 3.8 μM ABA (Table 2).

Table 2. Average number of cotyledonary stage embryos on various proliferation media. Evaluation 8 weeks after transfer of embryogenic callus clumps to proliferation medium. Experiment contained five replicates per treatment and was repeated thrice. Means ± SE. * - significant difference between NAA and BA, NAA+ BA and ABA, 2,4-D and BA, 2,4-D + BA and ABA.

NAA [μM]	2,4-D [μM]	BA [μM]	ABA [μM]	Number of embryos
0.27	-	-	-	3.2 ± 0.33
-	0.23	-	-	1.7 ± 0.24
0.27	-	2.2	-	5.4 ± 0.56
0.27	-	4.4	-	9.2 ± 0.53*
0.27	-	6.6	-	12.7 ± 0.64*
0.27	-	8.8	-	8.3 ± 0.59*
-	0.23	2.2	-	4.6 ± 0.81*
-	0.23	4.4	-	8.1 ± 0.55*
-	0.23	6.6	-	11.3 ± 0.57*
-	0.23	8.8	-	7.4 ± 0.52*
0.27	-	2.2	0.95	19.0 ± 0.49*
0.27	-	2.2	1.90	21.2 ± 0.92*
0.27	-	2.2	3.80	34.6 ± 0.78*
0.27	-	2.2	5.80	27.8 ± 0.59*
-	0.23	2.2	0.95	4.8 ± 0.71
-	0.23	4.4	1.90	9.2 ± 0.61*
-	0.23	6.6	3.80	13.3 ± 0.54*
-	0.23	8.8	5.80	16.2 ± 0.87*

In the present study, we found that the addition of BA and/or ABA to the medium containing low concentration of auxins (NAA or 2,4-D) favoured maturation of embryos from globular to cotyledonary stage. A significantly ($P < 0.001$) higher number of cotyledonary stage embryos occurred on proliferation medium containing ABA. Exogenously supplied ABA has been reported to regulate normal development of somatic embryos in embryogenic cultures (Nishiwaki *et al*. 2000). Enhanced production of cotyledonary stage embryos on ABA-enriched medium might be due to its ability to prevent precocious germination and accumulation of high level of storage proteins (Attree and Fowke 1993, Lecouteux *et al*. 1993).

Embryos germination: Germination of embryos was achieved after transfer of cotyledonary stage embryos to MSO medium or MS medium supplemented with 2.2 to 8.8 μM BA. Embryos germinated in 9 d on transfer to MS medium containing BA, which were characterized by simultaneous production of shoots and roots (Fig. 1*F*,*G*). With the various concentrations of BA used, embryo germination frequency ranged from 13.3 to 26.6 % (Table 3). It was enhanced significantly ($P < 0.05$ to 0.001) with the addition of GA_3 (0.29 to 5.8 μM) into MS medium containing 2.2 μM BA, and the highest

Table 3. Effect of BA and GA_3 on germination of embryos. Evaluation was recorded four weeks after transfer of cotyledonary stage embryos to germination medium. Experiment contained five replicates per treatment and was repeated thrice. Means ± SE. * - significant difference between MS and BA or MS and GA_3, ½ MS and BA or BA + GA_3.

Medium	BA [μM]	GA_3 [μM]	Germination frequency [%]
½ MS	-	-	0.0 ± 0.0
MS	-	-	6.7 ± 0.33
MS	2.2	-	13.3 ± 0.37*
MS	4.4	-	26.6 ± 0.43*
MS	8.8	-	17.6 ± 0.57*
MS	2.2	0.3	20.0 ± 0.57*
MS	2.2	1.4	33.3 ± 0.66*
MS	2.2	2.9	53.3 ± 0.66*
MS	2.2	4.3	66.7 ± 1.20*
MS	2.2	5.8	46.6 ± 0.42*
½ MS	4.4	-	13.3 ± 0.35*
½ MS	4.4	4.3	26.6 ± 0.43*

frequency (66.7 %) was observed on MS medium supplemented with 4.3 μM GA_3 (Table 3). Exogenously supplied cytokinin alone or in combination with gibberellin has been reported to promote the embryo maturation and subsequent development of embryos into plantlets (Lu and Vasil 1981).

The effect of various concentrations of sucrose and maltose was also studied on the germination of embryos. MS medium containing 4.4 μM BAP and 3.0 to 6.0 % maltose showed significantly ($P < 0.05$ to 0.001) higher frequency of embryos germination than the medium containing 3.0 to 6.0 % sucrose (Table 4). The best response (73.3 %) was observed on MS medium containing 5.0 % maltose. In *P. corylifolia*, MS medium enriched with maltose supported better germination of embryos than the medium containing sucrose. In alfalfa, Strickland *et al*. (1987) also observed that in comparison to sucrose, maltose serves to improve yield and development of somatic embryos when supplied at equal osmolarity. Chaleff and Stolarz (1981) suggested that influence of sugar might be due to the lowering of osmotic potential of the medium rather than due to its utilization as a carbon source. Nørgaard (1997) related the beneficial effect of maltose on embryogenesis to the fact that maltose is taken up directly from the medium, whereas sucrose is hydrolysed into monosacharides. The author also suggested that sucrose metabolise rapidly than maltose leading to hypoxia and ethanol accumulation in cells.

Table 4. Effect of sucrose or maltose on germination frequency of embryos. Evaluation was recorded four weeks after transfer of cotyledonary stage embryos to germination medium. Experiment contained five replicates per treatment and was repeated thrice. Means ± SE. * - significant difference between sucrose and maltose.

Sucrose [%]	Maltose [%]	Germination [%]
3.0	-	26.6 ± 0.33
4.0	-	46.6 ± 0.88
5.0	-	33.3 ± 0.67
6.0	-	20.0 ± 0.57
-	3.0	40.0 ± 0.67*
-	4.0	60.0 ± 1.00*
-	5.0	73.3 ± 0.67*
-	6.0	53.3 ± 0.88*

Cytological analysis: Cytological analysis was carried out on thirteen regenerated plants derived from the germination of embryos. Eleven plants showed haploid chromosome numbers and the remaining two plants showed to be diploid. Among the regenerated plants no mixoploidy, albinism or chlorophyll deficiency was observed. San and Gelebart (1986) also observed haploid and diploid cells among the regenerated plants derived from unpollinated ovaries of lettuce and sunflower. Chand and Basu (1998) have also reported variation in ploidy level in the regenerated plants derived from unpollinated ovaries of *Hyoscyamus muticus*. Among the regenerated plants studied, 25 % showed haploidy, 35 % diploidy and 40 % mixoploidy. Thomas *et al*. (1999) developed a reproducible protocol for the production of gynogenic haploids of mulberry (*Morus alba* L.) using ovaries from *in vitro* developed inflorescences. Out of the 20-gynogenic plants of mulberry examined cytologically, 12 showed haploid number of chromosomes and other 8 were aneuploids.

In conclusion, we have developed an efficient protocol for plant regeneration via embryogenesis from unpollinated ovaries of *P. corylifolia* by optimizing the growth regulators, type and concentration of sugar in the medium. The complete *in vitro* protocol for high frequency plant regeneration via embryogenesis may be helpful not only in production of large number of haploid plants which can be used in breeding program in order to enhance the valuable compounds of this plant species, but also in conservation and multiplication of this endangered plant species.

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