in-vitro Multiplication of *psoralea corylifolia* – an Endangered Medicinal Plant

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Abstract: *A protocol for in-vitro mass multiplication of Psoralea corylifolia plants through direct organogenesis from various seedling explants is described. The cotyledonary node explants developed the maximum number of adventitious shoots (5.0±0.5) per explant in 67% of cultures on MS containing 10μM 6-benzyl amino purine (BAP) within four weeks. Each shoot provided 2-3 culturable nodal segments. Shoots were further multiplied (5-6 shoots per explant) by culture of nodal segments. Shoots rooted on MS medium containing 5 μM indole-3 butyric acid with 100% efficiency. The plantlets showed 80% survival on transplantation in soil and were morphologically normal like the seed-raised plants.*

Keywords: *Psoralea corylifolia-micro-propagation from in vitro-raised shoots-nodal segments-6 benzyl amino purine-rooting.*

1. INTRODUCTION

Psoralea corylifolia L. is an endangered herbaceous medicinal plant of the family Fabaceae. It is distributed throughout the tropical and subtropical regions of the world (Jain 1994). The plant is well-recognized in Chinese and Indian folkloric medicines as laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic in febrile conditions (anonymous 1988). It has been specially recommended in the treatment of leucoderma, leprosy, psoriasis and inflammatory diseases of skin (Anonymous 1988). Recently, several phenylpropanoids like furano-coumarins (psoralen) and flavonoid diadzein have been reported in *P. corylifolia* (Bouque *et al.* 1998). Psoralen activates melanogenesis and is commonly employed in the cosmetics and sun tan preparation (Lane-Brown 1981, Kligman and Forlot 1989), Daidzein has been demonstrated to reduce breast cancer occurrence (Adlercreutz et al. 1991) and protect against colon cancer (Setchell et al. 1981). Antibacterial activity of *P. corylifolia* against *Staphylococcus aureaus* (Gaind et al.1965) and *Mycobacterium aurum* (Newton et al. 2002) has also been demonstreated.

Psoralea corylifolia is propagated by seeds which have very short span of viability. Seeds require pretreatment because of hard seed coat (Bhattacharjee 1998). Due to the exploitation of this medicinally important plant from the wild and natural habitat and inadequate efforts for cultivation, it is facing problem of extinction. There is an urgent need of *in vitro* propagation techniques for multiplication and conservation of genetic stock of this plant. A few reports on the *in vitro* plant regeneration via callus cultures (Saxena *et al.* 1997) or indirect somatic embryogenesis (Sahrawat and Chand 2001, Rout et al. 2001) are available. The establishment of callus (Bouque et al. 1998), cell suspension (Bourguad et al. 1989) and rhizogenic cultures of various species of *Psoralea* have also been achieved. However, the regeneration frequency in most of the reports is inefficient and also depends on the media composition and culture conditions. Morever, regeneration protocol based on the somatic embryogenesisis lengthy and difficult to reproduce. The callus cultures due to ageing or prolonged subscultures often show a progressive decline and even complete loss of morophogenic potential (Saxena et al. 1997). In this study, we report an efficient and a reproducible protocol for large scale multiplication of *P. corylifolia* via direct shoot organogenesis from different seedling explants on a simple culture medium containing only one cytokinin. The *in vitro* regenerated shoots have been further multiplied.

Materials and Methods

Plant material and preparation ofexplants

Seeds of *Psoraleacorylifolia* were obtained from local herbal medicine shop, New Delhi, India. Healthy and uniform seeds were treated with sulphuric acid for 45-50 min. and rinsed with double-distilled water several times till all the traces of acid were removed. The seeds were surface sterilized by immersion in 70% (v/v) alcohol for 1-2 min. and then with 0.1% (w/v) aqueous

mercuric chloride solution for 5-10 min. followed by rinsing with sterilized distilled water 4-5 times. The seeds were germinated aseptically on B_5 basal medium (Gamborg et al. 1968) containing 3% sucrose and 0.2% gelrite (Phytagel, Sigma, USA) in culture tubes (25mm × 150 mm) at 25°C under 16 hour photoperiod (80 μMms^{-2}) of cool-white fluorescent light. The entire cotyledon, hypocotyl (10 mm) and cotyledonary node (10 mm) excised from 7-day old seedings were used as explants. Cotyledonary node segments (10 mm) without both the cotyledons were excised from the seedlings by detaching of both the cotyledons and cutting both epicotyl and hypocotyl, approximately 4 mm above and below the nodal region and cultured in upright position with hypocotyl cut end slightly embedded in medium.

The entire cotyledons were cultured with proximal end sligthly embedded in medium while hypocotyl and stem node explants (10 mm) in upright position with cut end slightly embedded in the medium.

Culture medium

MS (Murashige and Skoog 1962) salts and vitamines containing 3% sucrose and 0.8% agar or 0.2% phytagel supplemented with different concentrations of BAP (0.25, 5.0, 10 µM) was used as culture medium. The pH of the medium was adjusted to 5.8 prior to addition of agar. Molten medium (20 ml) was dispensed in culture tubes (25 mm × 150 mm) and plugged with non-absorbent cotton wrapped in cheese cloth. The medium was autoclaved at 121°C for15 minutes.

In vitro shoot multiplication

The stem nodal segments (10 mm long) from the shoots regenerated *in vitro* from cotyledonary node explants were excised with a fine surgical blade and cultured in upright position with the cut end slightly embedded in medium. Callus which developed at the embedded end of cultured explants was isolated and cut into small pieces of approximately 50 mg (4 mm × 4 mm) and transferred to the same medium MS supplemented with BAP (2.5-10 µM) for shoot regeneration.

In vitro rooting and transplantation

Well-developed shoots (2-3 cm in length) were excised from the regenerating explants and cultured on full or half strength MS medium with 2% sucrose supplemented with IBA (2.5-5.0 µM) or IAA (0.30-0.06 µM) for rooting. Rooted shoots (plantlets) were removed from culture tubes and their roots were washed thoroughly with running tap water to remove agar. Plantlets were transferred to pots containing a mixture of sand: soil: vermiculite (1:1:1). Each plant was covered with polythene bag to ensure he high humidity for first few days and then humidity was reduced by making holes in the polythene bags.

Culture conditions

All cultures were maintained under 16 h photoperiod (80 $\mu Em^{-1}s^{-1}$) of cool-white fluorescent light at 25±2°C. Visual observations of cultures were taken every week and the effect of different treatments was quantified on the basis of percent cultures showing response and the degree of response per culture. For each treatment, 24 cultures were raised and each experiment was repeated twice. The data was subject to analysis of variance and significant treatment differences were selected by Newman-Keul's multiple range test (Brunning and Kintz 1977).

Results and discussion

The type of explants and dose of cytokinin is known to be critical for the multiple shoot induction. BAP has been found to be the most effective cytokinin for the multiple shoot formation (Gulati and Jaiwal 1994, Shiv Prakash *et al.* 1994, Sainiet al. 2003). Thus response of different seedling explants of mature seeds seeds to various concentrations of BAP for shoot regeneration was compared. All the explant types cultured on the MS basal medium did not regenerate shoots or roots. However, addition of BAP to the MS basal medium did not regenerate shoots or roots. However, addition BAP to the MS basal medium enlarged the explants considerably within 7 days and induced the multiple shoot buds at the proximal end of cotyledon (Fig. 1b), at the nodal regions of the cotyledonary node (Fig. 1e) and upper cut end of hypocotyl explants (Fig. 1g) within 28 days of culture.

The explants along with shoot buds were transferred to the hormone-free basal medium for shoot elongation. Some of the shoot buds elongated to normal adventitious shoots and attained a mean length of 2-3 cm after two weeks of culture and provided an average of 2 to 3 culturable nodal cuttings (Fg. 1c). The nodal segments (10 mm long) were cultured individually on the MS basal medium containing BAP (2.5-10 µM) for further multiplication (Figs. 1h, li). BAP induced multiple shoots in 100% of the explants. BAPat 10 µM induced a maximum of 5-6 shoots per nodal segment within four weeks (Table 1). Following this procedure, 25-30 shoots were obtained from single node within 60 days.

Table 1: Effect of different concentrations of BAP on multiple shoot regeneration from different seedling explants of *P. corylifolia**

Culture medium = MS

Explant type	BAP concentration (μM)	% shoot regeneration	No. of shoot per explant** (Mean ± S.E.)	Mean shoot length (cm) ± S.E.	Basal callus
Cotyledonary node	0.0	0	-	-	0
	2.5	100	3.0 ± 0.3a	2.2 ± 0.4	++
	5.0	100	3.8 ± 0.3a	1.4 ± 0.1	++
	10.0	66.8	5.0 ± 0.5b	1.1 ± 0.1	++
	0.0	0	0	-	-
	2.5	40	1.1 ± 0.9c	1.2 ± 0.4	++
Cotyledon	5.0	46.6	3.1 ± 0.5a	2.5 ± 0.5	+++
	10.0	100	1.2 ± 0.2c	1.3 ± 0.3	+++
	0.0	0	0	-	-
Hypocotyl	2.5	50	1.8 ± 0.5c	1.9 ± 0.6	+
	5.0	75	2.8 ± 0.3a	2.8 ± 0.4	++
	10.0	80	1.9 ± 0.6a	1.8 ± 04	++
	0.0	0	-	-	-
Stem node	2.5	100	1.4 ± 0.2c	2.6 ± 0.2	-
	5.0	100	3.2 ± 0.1a	2.1 ± 0.3	-
	10.0	100	5.5 ± 0.3b	1.8 ± 0.2	-

* Data recorded after six weeks culture No. of '+' = degree of callusing

** Mean value within columns followed by same letter are not significantly different according to Newman-Keul's multiple range test (P=0.05)

The regeneration frequency also varied with the concentration of the BAP and type of explant. The highest shoot regeneration frequency was observed from cotyledonary node followed by the cotyledon and hypocotyl explants. BAP at the concentration of 5 μM was found to be the optimal for induction of the maximum number of shoots from cotyledon, and 10 μM for hypocotyl and cotyledonary node explants, respectively. BAP concentration showed an inverse effect on the shoot length from the different explants. The higher concentrations of the BAP decreased the shoot length (Table 1). The amount of the whitish-green callus formed at the base of shoots developed and proximal end of the cotyledon was directly proportional to BAP concentration. The calli were isolated from the explants and were gorwn on the MS basal medium containing different concentrations of BAP. The call grew, but failed to differentiate into shoots on any of the BAP treatment.

The free hand sections of the regenerated explants revealed that the shoot developed directly from the explant. The callus developed at the base of the regenerated shoots.

Direct development of multiple shoots without intervention of callus phase probably occurred from the pre-existing meristem at proximal end of cotyledon and at nodal region of cotyledonary nodes on simple culture medium containing BAP as sole growth regulator. The hypocotyl, a non meristematic explant also directly produced multiple shoots *de novo* on a simple culture medium at a relatively shorter time compared to other studies where explants cultured on complex medium containing, cytokinin and auxin (Saxena et al. 1997, Rout et al. 2000) differentiate shoot buds through an intervening callus phase. The regeneration through direct shoot organogenesis take less time to raise plants (about four weeks for shoot bud induction and an additional two weeks for elongation) and the regenerants are free from somaclonal variations.

IN VITRO ROOTING AND TRANSPLANTATION

All the shoots on transfer to rooting medium developed prolific roots with lateral branching. IBA (5.0 μM)was found to be the best for inducing branched and thick roots at the base of 100% of the shoots (Table 2) (Fig. 1i). Though IAA also induced rooting in

Figure 1. *In vitro* regeneration of multiple shoots from seedling explants of *Psoralea corylifolia*, on MS medium containing BAP (a-c) Regeneration of multiple shoot buds (b) and shoots (c) from proximal end of mature cotyledon (a), (d-e) Shoots (e) development from cotyledonary node explant (d), (f-g) Regeneration of shoots (g) from hypocotyls explant (f), (h-l) Regeneration of shoots (i) from nodal segments (h) of *in vitro* raised shoots. (J) Induction of rots from *in vitro* regenerated shoot cultured on basal MS medium supplemented with Indole-3-Butyric acid (5.0 µM).
(i) A potted plant growing in soil and sand (photographed after 3 weeks of transplantation)

Table 2: Effect of different concentrations of IBA or IAA on *in vitro* rooting of excised regenerated shoots of *P. corylifolia**

Culture Medium	% Rooting of shoot	Nature of rooting
$^1/_2$ MS	50	One or two rootlets
MS + IBA (0 µM)	25	One or two roolets
MS + IBA (2.5 µM)	100	One or two rootlets
MS + IBA (5.0 µM)	100	Prolific and high root no.
MS + IAA (0.03 µM)	100	No direct vasulature with shoots
MS + IAA (0.06 µM)	100	No direct vasulature with shoots

100% of the cultures, a small amount of the callus at the cut end of roots was also observed, but this was not found suitable for survival of the plantlets in soil. However, the shoots rooted on IBA medium showed more than 80% of the survival on transplantation to soil and resumed growth (Fig. 1k) and developed flowers and pod. Similar effects of IBA were also observed in *Peganum harmala* (Saini and Jaiwal 2000) and *Vitex negundo* (Thiruvengadam) and Jayabalan 2000). The regenerated plants were morphologically normal like the plants raised from seeds.

Conclusions

The present study demonstrates that the nodal explants excised from *in vitro* regenerated shoots possess a high potential for rapid multiple shoot formation on a simple medium containing BAP as a sole plant growth regulator. The direct shoot multiplication can be exploited to multiple elite genotypes and also for developing *in vitro* strategies for the conservation and genetic transformation of this useful medicinal plant.

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References

(1) Adlercreutz, H., Y. Mousavi, M. Loukovaara, and E. Hamalainen, 1991: Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochhberg R.B. & Naftolin F. (eds.): THe New Biology of Steroid Hormones, 74, 145-154. Raven Press, New York.

(2) Anonymous, 1998: The wealth of India: A dictionary of Indian raw materials and industrial products, CSIR, New Delhi, India. 2, 116-118.

(3) Bhattacharjee, S.K. (ed.), 1998: In: Handbook of medicinal plants, pp. 287-288, Pointer Publishers, Jaipur, India.

(4) Bourgaud, F., C. Nguyen, and A. Guckert, 1995: *Psoralea species:* in vitro culture and production of furanocoumarins and other secondary metabolites. In: Bajaj Y.P.S. (ed.) Biotechnology in Agriculture and Forestry, **33,** 388-411. Medicinal and Aromatic Plants VIII Springer-Verlag, Berlin Heidelberg.

(5) Bouque, V., F. Bourgaud, C. Nguyen, and A. Guckert, 1998: Production of diazein by callus cultutres of *Psorales* species and comparison with plants. Plant Cell Tissue Org. Cult. **53,** 35-40.

(6) Bruning, J.L. and B.L., Kintz, 1977: In: Computational Handbook of Statistics, Scott, Foresman, Glenview, Ilinois.

(7) Gamborg, O.L., R.A. Miller, and K. Ojima, 1968: Nutrient requirement of suspension cultures of soybeam root cells. Exp. Cell Res. **50,** 151-158.

(8) Gaind, K.N., R.N. Dar, B.M. Chopra, and R.N. Kaul, 1965: Antihelmintic properties of the seeds of *Psoralea corylifolia,* Ind. J. Phram, **27,** 198.

(9) Gulati, A., and P.K. Jaiwal, 1994: Plant regeneration from cotyledonary node explants of mungbean *(Vigna radiata* L.) Wilczek. Plant Cell. Rep. **13,** 523-527.

(10) Jain, S.K., 1994: Ethnobotany and research in medicinal plants in India. Ethnobotanical Search New Drugs, **185:** 153-168.

(11) Kligman, A.M. and P. Forlot, 1989: Comparative photoprotection in humans by tans induced either by solar simulating radiation or after a psoralen-containing sunscreen. In: fitz-patrick, T.B., P. Forlot, M.A. Pathak, F. Urback (ed.): Psoralens, past, present, and future of photo-chemoprotection and other biological activities, 407-420, J. Libbey Eurotext, Paris.

(12) Lane-Brown, M.M., 1981: Australian field trial with bergamot oil-containing sun tan producs. In: Chan J., P. Forlot, C. Grupper, A. Meybeck, and A. Urback (ed.): Psoralens in cosmetics and dematology, 399-409, Pergamon Press, France.

(13) Murashige, T., and F. Skoog, 1962: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physoil, Plant, **15:** 473-497.

(14) Newton, S.M., C. Lau, S.S. Gurcha, G.S. Besra, and C.W. Wright, 2002: The evolution of forty-three plant species for *in vitro* anti-mycobacterial activities; isolation of active constituents from *Psoralea corylifolia and Sanguinaria Canadensis.* J. of Ethnophramacology **79:** 57-67.

(15) Rout, G.R., and P. Das, 2001: Studies on *in vitro* somatic embryogenesis of *Psoralea corylifolia* Linn. - An endangered medicinal plant. Gartenbauwissenschaft, **6:** 202-206.

(16) Rout, G.R., S. Samantaray, and P. Das, 2000: *In vitro* rooting of *Psoralea corylifolia* Linn: peroxidase activity as a marker. Plant Growth Reg. **30:** 215-219.

(17) Saharawat, A.K., and S. Chand, 2001: Continuous somatic embryogenesis and plant regeneration from hypocotyls segments of *Psoralea corylifolia Linn,* an endangered and medicinally important Fabaceae platn. Current Science **81:** 1328-1331.

(18) Saini, R., and P.K. Jaiwal, 2000: *In vitro* multiplication of *Peganum harmala* - an important medicinal plant, Ind. J. Expl. Biol, **38:** 499-503.

(19) Saini, R., Sonia, and P.K. Jaiwal, 2003: Stable genetic transformation of *Vigna mungo* L. Hepper via *Agrobacterium tumefaciens.* Plant Cell Rep. **21:** 851-589.

(20) Saxena, C., S.K. Palai, S. Samantaray, G.R. Rout, and P. Das, 1997: Plant regeneration from callus cultures of *Psoralea corylifolia* Linn. Plan Growth Reg. **22:** 13-17.

(21) Setchell, K.D.R., A.M. Lawson, S.P. Borriello, R. Harkness, H. Gordon, D. Morgan, D.N. Kirk, H. Adlercreutz, L.C. Anderson, and M. Axelson, 1981: Lignan formation in man-microbial involvement and possible roles in relation to cancer. Lancet **2:** 4-7.

(22) Shiv Prakash, N., D. Pental, and N.R. Bhalla, 1994: Regeneration of peagonpea from cotyledonary nodes via multiple shoot formation. Plant Cell Reg. **13:** 623-627.

(23) Thiruvengadam, M., and N. Jayabalan, 2000: Mass propagation of *Vitex negundo* L. - *in vitro.* J. Plant Biotech. **2:** 151-155.