



Development of an efficient *in vitro* regeneration protocol for rapid multiplication and genetic improvement of an important endangered medicinal plant *Psoralea corylifolia*

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ABSTRACT

Psoralea corylifolia linn. (Fabaceae) is an important herbaceous medicinal plant. The plant is well known for its different medicinal properties. Various parts of plant including roots and seeds are source of valuable alkaloids which have pharmaceutical importance. Three explants were compared to determine an efficient regeneration method for propagation of *Psoralea corylifolia*. Efficient and rapid *in vitro* seed germination has been established using B₅ basal medium with 0.7% agar. Good regeneration was obtained from 5 day old cotyledone node explant as compared to hypocotyls and cotyledone. 6-Benzylaminopurine (BAP) at 2.5 μM was found optimal for multiple shoot induction. Kanamycin at concentration of 35 mg/L caused complete bleaching of the non-transformed shoots. This concentration was found optimal for the selection of green putative shoots transformed by *Agrobacterium tumefaciens*. Indole-3-butyric acid (IBA) at 5.0 μM was found optimal for induction of roots approximately in 80% of cultures. The transformation was observed directly GUS staining of the explants. Transient GUS activity was detected after co-cultivation with *Agrobacterium tumefaciens* (EHA 105) carrying binary vector pCAMBIA 2301 which contained uid A gene as scorable marker for GUS expression and npt II as selectable marker for kanamycin resistance. Transient GUS blue staining was observed in transformed explants while non-transformed did not revealed any staining. Present study will help to propagate the medicinal plant for industrial herbal extraction of valuable pharmaceutical substances to be used in different diseases. This study will also help to execute further research on regeneration, transformation and hairy root induction of this medicinal plant.

Keywords: *Psoralea corylifolia*, medicinal plant, regeneration, cotyledone node, *Agrobacterium tumefaciens*, GUS expression

INTRODUCTION

Psoralea corylifolia linn. (Fabaceae) is a rare medicinal plant which is distributed throughout the tropical and sub-tropical regions of the world. *Psoralea* genus contains approximately 120 species which are distributed in South Africa, North America and Australia. It is also present in Asia, South America, North America and Mediterranean region [1]. Among the *Psoralea* plants known worldwide, *Psoralea corylifolia* is native to India. In many parts of India, it is cultivated as medicinal plant. Seeds and roots of this plant have been used successfully against several diseases. This endangered plant is well known for its medicinal properties in Chinese and Indian folkloric medicine [2]. It has been assigned as aphrodisiac, laxative, diuretic, anthelmintic, and diaphoretic in febrile conditions [2]. Seeds extract have been shown to display antibacterial activity against *Staphylococcus aureus* [3]. Different valuable

compounds including furanocoumarin, glycoside like psoralen, isopsoralen, psoralidin, isopsoralidin, isovachinin and monoterpenoid phenols etc. are found in *Psoralea corylifolia* [4].

The conventional method for propagation of *Psoralea corylifolia* is through seed germination. But this is not adequate because of hard seed coat which requires pretreatment and short period of seed viability. Due to these drawbacks, there is a great need for micro-propagation of this plant along with genetic stock conservation. Tissue culture offers an alternate method for rapid multiplication of desirable clones. Earlier studies described regeneration of *Psoralea corylifolia* by somatic embryogenesis and hypocotyls explants [2]. Recently, regeneration and micro-propagation of *Psoralea corylifolia* has been reported [5]. In this study, authors reported direct regeneration from hypocotyls explants. In regeneration of any plant, the regeneration efficiency has been found to depend on genotype, type of explant, media composition and culture conditions. In spite of earlier studies, more efficient and rapid *in vitro* propagation techniques are required for the genetic transformation and conservation of this medicinal plant. The present study describes an efficient method for regeneration and propagation of the *Psoralea corylifolia*. An efficient seed germination protocol based on acid treatment has been performed followed by comparison of regeneration from three different explants i.e. hypocotyl, cotyledon and cotyledonary node has been described in this study. Further, genetic transformation of the cotyledonary node explant (producing higher number of healthy shoots) was attempted using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

MATERIALS AND METHODS

Plant material, bacterial strain and vector

Fresh seeds of *Psoralea corylifolia* were procured from Department of seed technology, Chaudhary Charan Singh Haryana Agriculture University, Hissar. The disarmed *A. tumefaciens* strain EHA105 harboring a binary vector pCAMBIA2301, which contains a β -glucuronidase (*GUS*) gene (*uidA*) and a neomycin phosphotransferase gene (*nptII*) both driven by cauliflower mosaic virus (CaMV)35S promoter was used for transformation (Fig. 1). The *uidA* gene contains an intron in its coding region to ensure that the observed *GUS* activity occurs in the plant cell and is not due to presence of residual *Agrobacterium* cells.

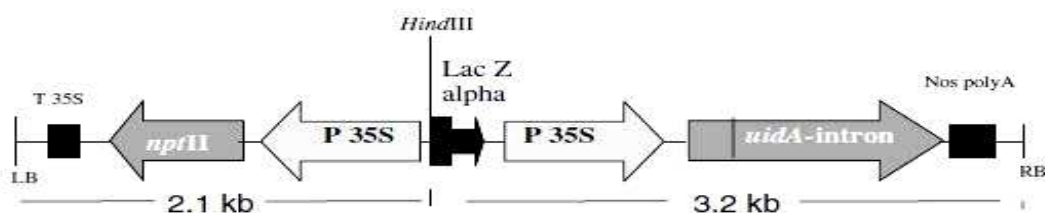


Fig. 1. Schematic representation of the T-DNA (5.2 kb) of pCAMBIA2301 containing the *uidA* genes and *nptII* genes (not drawn to scale).

2.2. Seed germination, Explants preparation and Plant regeneration

2.2.1. Selection of germination medium

Healthy and uniform seeds were treated with concentrated sulphuric acid for 40-45 minutes and washed 6-7 times with distilled water to completely remove the traces of acid. Seeds were surface sterilized for 2 minutes in 70% (v/v) alcohol followed by treatment with aqueous mercuric chloride (0.1%, w/v) for 5-10 minutes. Seeds were then rinsed with sterilized distilled water (4-5 times). The seeds were cultured on different basal medium MS [6], B₅ [7], MSB₅, and water (each media supplemented with 3% sucrose and 0.7% agar) at 25°C under 16 hours photoperiod of cool-white fluorescent light for germination. Seeds were also allowed to germinate in 250 ml flask containing 50-100 ml of autoclaved distilled water at 25±2°C in an orbital shaker at 200 rpm.

Explants for plant regeneration

The entire cotyledons, hypocotyls and cotyledonary nodes excised from *in vitro* raised 5 day old seedlings were compared for plant regeneration. All explants were excised with sterilized fine surgical blade.

Optimization of 6-benzylaminopurine (BAP) concentration

All explants were cultured in MS basal media supplemented with various concentrations of BAP (0.5-10.0 μ M) to find its optimal concentration for multiple shoot regeneration. The explants were cultured for 4 weeks. All cultures were maintained at 25±2°C under 16 hour photoperiod of cool white fluorescent light of 80 μ E m⁻² s⁻¹ intensity.

Optimization of indole-3-butyric acid (IBA) concentration

After 4 weeks, individual shoots (2-3 cm) were excised from the base and cultured on MS basal medium supplemented with different concentrations of IBA (2.5-5.0 μ M) with sucrose (2%, w/v) for root induction in culture tubes (25 x 150 mm). All cultures were maintained as described in section optimization of BAP concentration.

Acclimatization of rooted shoots

The rooted shoots were removed from the culture test tubes and roots were washed extensively but gently with distilled water. The plantlets were then transferred to the plastic pots containing a mixture of soil, sand and cow dung manure (1:1:1). The pots initially were covered with polythene bags to insure high humidity around the plantlets during first few days. Plantlets were watered after every 3 days. Subsequently, humidity was reduced gradually by removing the polythene bags for varying time interval before transferring the plants to soil.

Optimization of Kanamycin concentration for selection of transformed shoots

An optimal concentration of kanamycin was for selection of transformed shoots by culturing the non-transformed (control) explants. The explants were cultured on shoot regeneration medium (MS + 2.5 μ M BAP) supplemented with different concentrations of kanamycin (25, 35, 50 mg/l). The cultures were transferred to the same fresh medium containing same level antibiotic after two weeks. The explants were cultured for 4 weeks and scored for frequency of regeneration.

***Agrobacterium tumefaciens* mediated genetic transformation**

The *Agrobacterium tumefaciens* (EHA 105) was grown in YEM medium containing rifampicin (10 mg/l) and kanamycin (50 mg/l) for 12 hours at 27°C. The bacterial culture was harvested and resuspended in MS media supplemented with 2.5 μ M BAP and 20 mM acetosyringone.

The cotyledonary nodes were excised from *in vitro* raised 5 day old seedling with help of sterilized blade in laminar flow. The explants were gently stabbed 4-5 times with a sterilized fine needle and immersed in bacterial suspension at 25°C for 25 min with occasional shaking. The explants were co-cultured in petridishes lined with filter paper moistened with MS medium containing 2.5 μ M BAP. Further, the explants were washed with sterilized distilled water, dried and then cultured on solid MS media supplemented with IBA and cefotaxime (500 mg/l). The rooted shoots were removed and transferred to plastic pots as described earlier in manuscript.

Hairy root induction with *Agrobacterium rhizogenes***Genetic transformation of *Psoralea corylifolia* with wild *Agrobacterium rhizogenes***

Agrobacterium rhizogenes was inoculated and harvested as described for *Agrobacterium tumefaciens*. The explants (cotyledonary nodes) were submerged in bacterial suspension for 25 min at 25°C under constant shaking. The explants were co-cultured in petridishes as described for *Agrobacterium tumefaciens*. After 3 days incubation explants were washed and cultured on MS and B₅ media containing cefotaxime (500 mg/l) for hairy root induction.

Genetic transformation of *Psoralea corylifolia* with pCAMBIA harboring *Agrobacterium rhizogenes*

Competent cells of *Agrobacterium rhizogenes* were prepared by CaCl₂ (20 mM) method. The DNA vector pCAMBIA 2301 was extracted from *Agrobacterium tumefaciens* and transformed in *Agrobacterium rhizogenes* using standard transformation protocol. Thereafter, the explants (cotyledonary nodes) were incubated with *Agrobacterium rhizogenes* harboring pCAMBIA and wild type Ri plasmid for 25 min at 25±2°C. The explants were co-cultured for 3 days and shifted to MS and B₅ medium containing cefotaxime (500 mg/l) for hairy root induction.

Enzyme assay

The GUS activity in tissues was determined by histochemical assay as described by Jefferson (1989). The transformed and non-transformed explants were immersed in freshly prepared X-gluc (5-bromo-4-chloro-3-indolyl- β -glucuronidase) solution and incubated overnight at 37°C. The staining solution was removed and explants were washed with ethanol. The explants were examined under microscope.

Statistical analysis

The cultures were observed regularly and results were analyzed for variance and standard error tests to ensure the accuracy of the experiments.

RESULTS AND DISCUSSION

Seed germination

Seed germination in plants is a critical period determining further perpetuation of the species in natural habitat [8]. Seeds of *Psoralea corylifolia* have hard seed coat and also have short viability period which generally results in poor germination. Conventionally, *P. corylifolia* is propagated by seed germination but the conventional method of propagation of *P. corylifolia* through seeds is not adequate and efficient, as seeds need pretreatment because of hard seed coat [9]. Therefore, seeds were subjected to acid treatment. Results are predicted in Table 1. Amongst the different media used for germination of seeds, water soaked seeds showed highest percentage (85%) of germination. Water soaked method was followed by B₅ medium which gave 36% germination. Other methods used for seed germination were found non-efficient.

Table 1: Germination of seeds of *P. corylifolia* on different medium

Sr. No.	Medium used	Number of seeds per treatment	Seeds germinated	Germination period (days)	Percentage of seed germination
1	Water-agar	25	6	5-6 days	22
2	MS Basal	25	6	4-5 days	22
3	B ₅ Basal	25	9	3-5 days	36
4	MS salts + B ₅ vitamins – Basal	25	2	5-7 days	8
5	Water soaked seeds	100	85	3-4 days	85

Plant regeneration (*in vitro*) through multiple shoot formation**Cotyledonary node**

The explant could not regenerate any shoot on MS basal medium even after prolonged culture. However, addition of BAP to MS media induced a variable amount of callus at the basal cut end of the explant followed by differentiation of multiple shoots from nodal region with in 28 days of culture. Increase in concentration of BAP upto 2.5 μ M increased the number and length of the shoots. Further increase in BAP concentration resulted in decreased frequency of shoot regeneration (Table 2). Although, highest percentage of regeneration of was obtained with 0.5 μ M with an average length of 1.3 cm but the average number of length per explant was very low. BAP at 2.5 μ M found optimal for multiple shoot regeneration (Table 2). The presence of BAP was found an essential condition for multiple shoot induction. Plant regeneration through cotyledonary node has been shown in Fig. 2. Earlier reports also described that BAP induced multiple shoot regeneration from cotyledonary node explant of legumes [10;11].

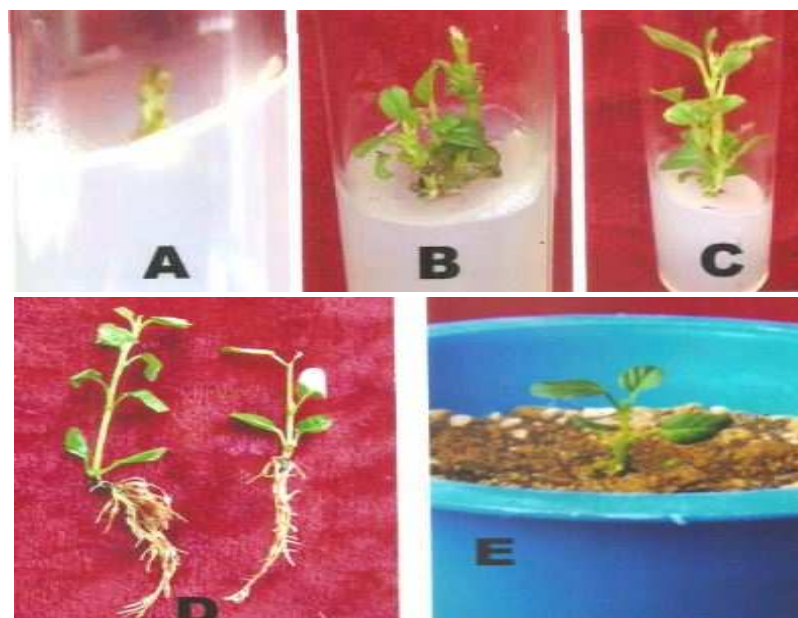


Fig. 2: Plant regeneration (*in vitro*) through multiple shoot formation from cotyledonary node explants. A, Explant at time of culture; B, Multiple shoots regeneration from cotyledonary node on MS +BAP (2.5 μ M) and kanamycin (35 mg/l) with cefotaxime (500 mg/l); C, Green putative transformed shoots on rooting medium (MS+ IBA) containing cefotaxime; D, Entire regenerated plantlets; E, Regenerated plants transferred to pot

Table 2: Effect of different concentrations of BAP on direct multiple shoot regeneration from cotyledonary node explants of *P. corylifolia*

BAP concentration (μM)	Percentage of regenerating culture	Average number of shoots per explant (mean \pm S.E.)	Average length (cm) of shoots per explant (mean \pm S.E.)
0.0	Nil	Nil	Nil
0.5	75	1.0 \pm 0.64	1.3 \pm 0.57
1.0	71	1.2 \pm 0.38	1.5 \pm 0.50
2.5	53	5.0 \pm 0.35	2.5 \pm 0.21
5.0	40	2.3 \pm 0.68	1.1 \pm 0.60
10.0	31	2.0 \pm 1.01	0.8 \pm 0.04

Hypocotyl

The hypocotyl segment around 0.7 cm in length was excised from 5 day old seedling and cultured in up right position. The explant showed shoot generation on MS medium supplemented with different concentration of BAP (Table 3). BAP at 2.5 μM resulted in good regeneration percentage (Table 3). Further increase in BAP concentration did not affect the percentage of regeneration. Addition of BAP to medium induced multiple shoot regeneration from non-meristemic hypocotyl explants of *P. corylifolia*. The percentage of regeneration was found lower as compared to cotyledonary node. Therefore, it is concluded that cotyledonary node is a better explant for regeneration of *P. corylifolia*.

Table 3: Effect of different concentrations of BAP on direct multiple shoot regeneration from hypocotyl explants of *P. corylifolia*

BAP concentration (μM)	Percentage of regenerating cultures	Average number of shoots per explant (mean \pm S.E.)	Average length (cm) of shoots per explant (mean \pm S.E.)
0.0	Nil	Nil	Nil
1.0	30	1.0 \pm 0.33	0.7 \pm 0.24
2.5	49	3.2 \pm 0.59	1.7 \pm 0.58
5.0	40	2.0 \pm 0.94	1.1 \pm 0.62

Cotyledon explant

The cotyledon explants cultured on MS basal medium failed to develop any shoot even after six weeks of culture. The explants cultured on MS supplemented with BAP resulted in swelling of the explants followed by callus formation at the embedded proximal ends within 4 weeks. Differentiation of multiple shoot buds was observed from the callus only on 2.5 μM BAP (Table 4). Other concentrations resulted in caulogenesis. The percentage of regeneration was quite low as compared to regeneration from cotyledonary node and hypocotyl. Maximal regeneration efficiency and percentage was obtained with cotyledonary node. Therefore, cotyledonary node explant was selected for further studies.

Table 4: Effect of different concentrations of BAP on direct multiple shoot regeneration from cotyledon explants of *P. corylifolia*

BAP concentration (μM)	Percentage of regenerating cultures	Average number of shoots per explant (mean \pm S.E.)	Average length (cm) of shoots per explant (mean \pm S.E.)
0.0	Nil	Nil	Nil
1.0	10*	1.0 \pm 0.33	0.7 \pm 0.24
2.5	22*	3.2 \pm 0.59	1.7 \pm 0.58
5.0	40	2.0 \pm 0.94	1.1 \pm 0.62

*Callus formation only.

Rooting of *in vitro* regenerated shoots and their acclimatization

The regenerated transformed shoots from cotyledonary node were transferred MS basal medium containing IBA with 2% sucrose. The shoots developed thick and branched roots on IBA containing medium with in 15 days of culture. IBA at 5.0 μM was found to be optimal for inducing roots in 80% of the cultures (Figure 1). The rooted shoots were transferred to pots and covered with polyethylene bags. The humidity was gradually reduced by making holes in the bags. All the regenerated plants showed normal morphological characteristics. Eighty percent of plantlets survived and showed normal growth after four weeks of transplantation.

Selection of Kanamycin for the putative transformants

The cotyledonary node explants excised from 5 day old seedlings were on MS basal medium containing BAP (2.5 μ M) and different concentration of kanamycin (0, 25, 35, 50 mg/l). Kanamycin at concentration higher than 25 mg/l caused drastic reduction in shoot regeneration (Table 5). Kanamycin at higher concentrations caused complete inhibition of shoot regeneration (Table 5). Kanamycin at 35 mg/l did not impair the shoot regeneration but completely bleached the shoots regenerated from the non-transformed (control) explants. Kanamycin has been found effective in recovery of transformants in a number of legume crops like *Cicer arietinum* [12] *V. radiata* [13] and *V. mungo* [10].

Table 5: Effect of different concentrations of kanamycin on shoot regeneration from cotyledonary node explants of *P. corylifolia*

Culture medium	BAP concentration (μ M)	Kanamycin concentration (mg/l)	Percentage explants regenerated shoots
MS	2.5	Nil	66
MS	2.5	25	71
MS	2.5	35	67
MS	2.5	\geq 50	20

Genetic transformation with *Agrobacterium tumefaciens*

The cotyledonary node explant excised from 5 day old seedling were transferred to selection medium after co-culture with *Agrobacterium tumefaciens*. MS medium with BAP (2.5 μ M), kanamycin (35 mg/l) and cefotaxime (500 mg/l) was used for selection of green putative transformed shoots. The frequency of recovery of green putative transformed shoots was very low due to inefficient targeting of the transgene into the explants. An extra wounding treatment of explants at the nodal region prior to their inoculation in Agro-suspension was carried out to enhance the frequency of transformants. Pre-wounding treatment is essential because it release phenolic inducer of Agro *vir* genes, disrupt tissue organization such that *de novo* shoot induction can occur near wounded surface and stimulate host DNA replication for T-DNA integration. Out of 60 cotyledonary node explants inoculated with *Agrobacterium tumefaciens*, 45 green shoots were recovered on selection medium. Among these, 36 shoots formed roots on rooting medium. Though, hypocotyl and cotyledon explants were also studied for genetic transformation but the cotyledonary node explant was found most susceptible for *Agrobacterium tumefaciens* mediated genetic transformation. Cotyledonary node has also been reported adventitious explant for *Solanum lycopersicum* L. regeneration [14].

GUS activity

The transformation was quantified directly by observing the GUS blue staining in the explants. Transient GUS activity was observed in cotyledonary node after co-culture with *Agrobacterium tumefaciens* (EHA 105). The explants treated with *Agrobacterium rhizogenes* containing wild type Ri-plasmid and binary vector pCAMBIA 2301 that harbored *uid A* gene as a scorable marker with an intron in its coding region for GUS expression and *npt II* gene as selectable marker for kanamycin. After 3 days of co-culture, the explants were washed and immersed in freshly prepared X-gluc. (5-bromo-4-chloro-3-indolyl- β -glucuronidase) solution and incubated overnight for at 37°C. The explants were decolorized using ethanol. Transient GUS expression was observed in transformed explants (Figure 2A). The non-transformed (control) explants did not show any blue colour when stained with X-gluc (Figure 2B).

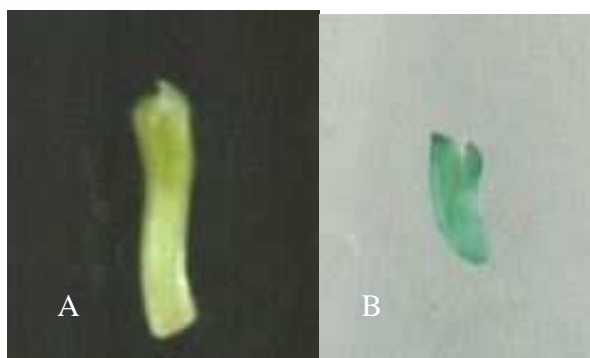


Figure 2: (A) Non-transformed cotyledonary node explant without GUS expression, (B) Cotyledonary node treated with *Agrobacterium tumefaciens* showing transient GUS expression

In Indian system, about 1500 medicinal plants have been identified, out of which 500 species are mostly used in the preparation of drugs [15]. *Psoralea corylifolia* is also among the important medicinal plants. The studies on regeneration and transformation with *Agrobacterium tumefaciens* has provided an important insight for micropropagation of this plant. Regeneration of important plants may be a constraint for genetic engineering of plants [16]. Therefore, attempt has been made to develop the efficient regeneration of a important medicinal plant and its genetic engineering.

CONCLUSION

The present study has shown *in vitro* regeneration from various explants of *Psoralea corylifolia*. An efficient *in vitro* regeneration protocol for *Psoralea* has been developed. The seed germination efficiency was greatly enhanced when soaked with water. The shoot regeneration from cotyledonary node was found efficient and can be exploited to multiply this endangered medicinal properties. Since, cotyledonary node gave maximal number of shoots at 2.5 μ M BAP, therefore, it is best explant for propagation of *Psoralea corylifolia*. Rapid and reproducible *in vitro* regeneration protocol has been established from 5 day old cotyledonary node explants. BAP (2.5 μ M) was found effective for shoot induction while IBA (5.0 μ M) was found optimal for root induction. Kanamycin at 35 mg/l caused complete bleaching of the non-transferred shoots. This concentration was found optimal for selection of green putative shoots transformed with *Agrobacterium tumefaciens*. Present study will provide important insight for further research on conservation of this important but endangered medicinal plant.

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