RESEARCH PAPER | Pharma | Volume : 4 | Issue : 4 | Apr 2014 | ISSN - 2249-555X

Novel and Simple Method for *in Vitro* Production of Psoralen via Cotyledonary Culture of An Endangered Leguminous Taxon- *Psoralea Corylifolia* Linn.

KEYWORDS: Cotyledon, Callus, Psoralen, *Psoralea corylifolia* Linn. and HPLC

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ABSTRACT *First time, a novel and simple protocol has been successfully developed for in vitro production of psoralen through rapid and recurrent callus cultures from one week old cotyledon of Psoralea corylifolia Linn. Amongst the various auxins and cytokinins tried alone or in combinations, 10 μM 2,4-dichlorophenoxyacetic acid (2, 4-D) supplemented to Gamborg et al. (1968) basal medium (B5) was optimum for inducing profuse amount of callus in cent percent cultures of cotyledon. The cotyledon calluses were subcultured continuously at an interval of 35-40 days on 5μM 2, 4-D medium up to two year and good amount of callus proliferation was achieved in cent percent cultures. However, some cultures were maintained under optimum cultural condition on B5+5μM 2, 4-D+5μM BA with 0.5% polyethylene glycol (w/v) up to 3 months. These callus cultures showed the maximum psoralen content of 16.15 mg/g FW. Psoralen has been detected time to time from calluses of different time durations grown on different type of culture media under optimum cultural conditions through HPLC by using calibration curve of standard psoralen. Psoralen estimation was also done for mature seeds samples collected from various localities of central India.*

Introduction:

<u>*Psoralea corylifolia*</u> (Linn.) is enlisted as rare and endangered leguminous taxon (Jain, 1994). Four species are found in India of which <u>*P. corylifolia*</u> is medicinally valuable plant (Anonymous, 1989; Bhattacharjee, 1998). It is used in indigenous medicine as laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic and specially recommended for the treatment of vitiligo , leprosy, psoriasis and inflammatory diseases of the skin in ayurvedic as well as allopathic system of medicines(Anonymous,1989). The medicinal value is due to the presence of furanocoumarin especially a psoralen in seeds that cost around US $ 4634/- per gram (Sigma-Aldrich Chemical Company, USA). In recent time, it is compulsory to have some alternative source of these seeds that produce the highly important bioactive compound known as Psoralen.. Till date, there is no report of <u>*in vitro*</u> production of Psoralen , therefore, the present study becomes globally novel, simple and reproducible in which cotyledonary callus cultures were used for the continuous production of psoralen at commercial/ industrial scale.

Materials and methods:

(i) Source of seed materials:

Mature and immature seeds of <u>*Psoralea corylifolia*</u> Linn. has procured from different areas/ populations of central part of India .

1. Pharmacopoeial Laboratory for Indian Medicine, Kamla Nehru Nagar, Ghaziabad, U.P., India.
2. Botanical Garden, University of Jamia Hamdard, Delhi, India.
3. National Bureau of Plant Genetic Resources, Pusa Campus, Delhi, India Accessions No. IC-111249, IC-111228, IC-111238
4. National Botanical Research Institute, Lucknow, India.
5. Pratap Nursery, Dehradun, Uttrakhund, India.
6. Kharibawali, Delhi, India.

(ii) Explants:

Cotyledonary callus cultures was developed using the 7 day old cotyledonary explants taken from above mentioned sources.

(iii) Sterilization:

a) Glasswares, instruments, etc.: Glasswares, instruments (wrapped in aluminium foil) and nutrient media in glass vessels were sterilized by autoclaving them at 1.06 Kg cm^{-2} pressure for 15-20 min at 121^0C.

b) Seeds: Mature and immature seeds were washed under running tap water for 10 min in a beaker. They were then immersed in 1% (v/v) germicide-detergent Cetrimide solution (Nicholas Piramal India Ltd., Bombay) and stirred vigorously on magnetic stirrer for 10 min. These were rewashed under running tap water to remove any traces of Cetrimide. After pouring out excess water, the seeds were treated with 0.1% Bavistin and 0.1% Amoxicillin solution for 10-15 min. Later, washing with tap water, seeds were soaked in 15% concentrated sulfuric acid (H_2SO_4) for 20 min or in hot water at 50^0C for 3 hours in order to soften the hard seed coat. After decanting the acid or hot water, seeds were rinsed with autoclaved distilled water 4 or 5 times. Subsequently, the seeds were treated with freshly prepared 0.1% (w/v) $HgCl_2$ solution (Merck, India) for 10-15 min, keeping the vessel on the shaker with constant stirring. Finally, the seeds were given 4 or 5 washings with sterilized distilled water under laminar flow cabinet and inoculate onto medium.

(iv) Culture media:

Cotyledon were implanted on different basal media, viz. Knop's (Knop, 1865), MS (Murashige and Skoog, 1962,), B5 (Gamborg et al., 1968), and NN (Nitsch, 1969) to assess their germination potential. Stock solution of these media were prepared using analytical grade (AR) salts (Qualigens, Merck, Hi-media, SRL and BDH) and stored in reagent bottles in the refrigerator.

The basal media were used alone or in combination with various plant growth regulators such as (a) cytokinins, viz. BA, 2iP, Kn and /or (b) auxins, viz. NAA and 2,4-D procured from Sigma-Aldrich Chemical Company, USA. . In addition, adjuvants like adenine sulphate, picloram, polyethylene glycol and coconut water were also supplemented to the medium. The thermolabile growth regulators, and antibiotic (ampicillin) were filter sterilized using 0.22 mm millipore filter and then added to the autoclaved and cooled (45-48^0C) medium under a laminar flow cabinet

As a source of carbon, sucrose (DCM, Daurala) at 3% concentration (w/v) were used in all experiments unless mentioned specifically. The media were gelled with 0.8% agar (Qualigens, Bombay) for morphogenesis. The pH of the

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media were adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving.

In some experiments, cultures were also kept under different light conditions, temperature range, culture vessels, pH, orientation and size of cotyledonary explants in order to see the effect of Physico-chemical conditions on callogenic potential of explants.

(v) Raising and incubation of cultures:
Cotyledonary callus were reared in test tubes and jars or conical flasks. Cultures were incubated in the culture room at 25±3^{0}C temperature and 55±5% relative humidity under white fluorescent light (400-450 mW cm^{-2}) emitted by 40 W Philips tubes fixed to the shelves of trolleys on which cultures were kept under 16 h light/ 8 h dark.

(vi) Subcultures:
The cultures were subcultured at an interval of 25-30 d. In a few experiments, quick (at an interval of 15 d) and late subculturing (at an interval of 2 or 3 months) were carried out.

(vii) Recording of data:
Observations were recorded after every week till thirty day. However, for some experiments final data were recorded after 60 d. The morphogenic responses of explants were observed in terms of:

a) Caulogenesis:
1. Percentage of explants producing single and multiple shoots,
2. Average number of shoots per explant, and
3. Average shoot length.

b) Callogenesis:
1. Percentage of callus,
2. Colour and nature of callus, and
3. Amount of callus recorded by weighing or on visual basis as nil (-), scanty (+), moderate (++), good (+ + +), and profuse (+ + + +).

c) Statistical analysis:
The percentage of callus and amount of psoralen through HPLC have been represented as mean values. The mean values calculated on the basis of a minimum of 12 replicates in each experiment, repeated once or twice or thrice depending on the experiments. The data have been statistically analysed using Analysis of Variance (ANOVA) through SAS. Between the treatments, the average figures followed by the same letter were not significantly different at $p \leq 0.05$ level.

(viii) Suspension culture:
The preparation of suspension culture were done for extraction of the Psoralen contents. The cultures were also kept for suspension culture in the Orbital shaking incubator cum BOD incubator (Narang Scientific NSW-256, India).

(ix) Psoralen estimation:
a) Sample preparation for Psoralen estimation: The fresh samples (1g, each) of calluses and seeds were weighed. The extracts were prepared in dark condition. The tissues were homogenized using pestle and mortar with 20 ml of solvent methanol and kept for 48 h in the Orbital shaking incubator cum BOD incubator (Narang Scientific NSW-256, India) under dark. This mixture were transferred into centrifuge tube and centrifuged for 45 min at 12,000 rpm at 4^{0}C temperature. The pellet were discarded and the supernatant were filtered using 0.4 µm millipore filter and this will be used as sample solution. The presence of psoralen in the methanolic extract was confirmed by co-chromatography of samples and Psoralen standard (Sigma-Aldrich Chemical Company, USA) using TLC. 20 µl sample solutions were injected into apparatus for HPLC analysis.

b) Standard preparation: Precisely weigh 1.5 mg of pure psoralen powder (Sigma Aldrich Chemical Company, USA), dissolve in 1.5 ml of solvent (methanol). From the stock solution, various dilutions were made so as to have psoralen of concentration in the range of 10-50 µg. These were analyzed independently by HPLC and standard curves were plotted between concentrations and peak areas.

c) TLC : Methanolic extract of each sample along with authentic sample were resolved on glass sheets coated with Silica gel 60F254 (20x20 cm, Merck, Germany). The plates were developed in an air tight chromatographic chamber saturated with 200 ml of chloroform solvent system. The plates were activated at 50^0 C in an oven. The Rf values were calculated. The developed chromatogram was visualized under UV light in UV Cabinet (Matrix, Delhi). The brown fluorescence observed, confirmed the presence of Psoralen in samples.

d) HPLC: The reverse phase HPLC (Waters, USA) equipped with detector and printer operated under the following parameters:

Column	**:C^{18} (LichroCART 150-4.6 Purospher Silica 5 µm, Merck, USA)**
Column Packing	**:Purospher Silica**
Solvent	**:water: methanol (30:70)**
Injection volume	**: 20 µl**
Flow rate	**: 1 ml/min**
Detection at	**: UV 251 nm**

The area of the relevant peak of each sample was inter plotted with the standard curve to determine the quantity of Psoralen (in mg/g fresh weight of sample). At least three samples from each treatment were analyzed.

Results and Discussion:
***In vitro* morphogenic response:**
Germination of the seeds has been encountered as one of the major problem while establishing primary cultures in *P. corylifolia*. During the present investigation, the seeds were initially scarified by treating them with 15% conc. H_2SO_4 for 20 min. in order to enhance germination. This treatments improved percentage of seeds germination only up to 30 percent. However, no germination was seen in unscarified seeds as reported earlier (Shukla, 1972; Mitter et al., 1995; Gupta et al., 1997; Bhattacharjee, 1998). The optimum seed germination was achieved on absorbent cotton layer in petridish with autoclaved distilled water alone (Fig.1A). Whereas in some experiments, the variety of media were tried i.e. B5, MS, Knop's and NN but no improvement in seed germination was observed.

Seed collected from different regions of central part of India showed that Ghaziabad sample were the best in inducing good amount of callus in cent percent cultures. Among the different concentrations (0.1, 1,5 & 10 µM) of auxins (NAA and 2,4-D) tried, 2,4-D was significantly best in terms of callogenesis when augmented with B5 medium. Of all the combinations tried, 10 µM 2,4-D was the optimum where cent percent cultures induced profuse amount of callus which is brownish-yellow, nodular and soft after one month (Fig.1B;Table 1). These callus cultures were later maintained and subcultured continuously at an interval of 35-40 d on B5+5 µM 2,4-D medium upto two years and it was found that amount of of callus proliferation and yield of Psoralen increased with the age of cultures (Fig.2).

Table 1: Callogenic response of cotyledon explants of *P.corylifolia* on B5 medium augmented with 2,4-D. Data were recorded after 30 d. Experiment was repeated thrice giving consistent results.

2,4-D concentration (µM)	%age of callus** (%)	Amount of callus*	Nature and colour of callus
0	___	____	____
0.1	5.55^{d}	+	Nodular, soft and whitish-yellow
1	55.55^{c}	++	Nodular, soft and yellowish-brown
5	88.88^{b}	+++	Nodular, soft and brownish-yellow
10	100^{a}	++++	Nodular, soft and brownish-yellow

*** Relative amount of callus: Nil(-); scanty (+); moderate(++); good(+++); profuse(++++).**
**** Values in a column followed by the same superscript are not significantly different as determined by SAS at p≤0.05.**

In order to improve the amount of psoralen in the callus the cotyledonary explants were further cultured on media B5+ 5 µM 2,4-D with different concentrations (0.1, 1,5 & 10 µM) of cytokinins (BA, Kn and 2iP). In addition, adjuvants like adenine sulphate, picloram, polyethylene glycol and coconut water were also supplemented to the medium. Among these different media concentrations tried, the callus cultures grown on B5+5µM 2, 4-D+5µM BA with 0.5% polyethylene glycol (w/v) was the best for *in vitro* production of psoralen (Fig 1C; Fig.2). To standardized the optimal physico-chemical conditions ,the effect of various parameters, i.e., light, temperature, orientation of explants, size of explants and different types of culture vessels on callus growth and psoralen yield were also investigated. The optimal culture condition for maximum psoralen yield along with good callus growth was found to be 16 h light/ 8 h dark, 28^{0}C temperature, abaxial orientation of cotyledonary explants with 0.5 mm size and conical flask as culturing vessel (Fig.1D).Cotyledonary callus suspension was also maintained for two month on B5+ 5 µM 2,4-D liquid medium in BOD cum incubator shaker (Narang Scientific, India) under optimum cultural conditions but it does not significantly improve the amount of callus formation and yield of psoralen (Fig.2).

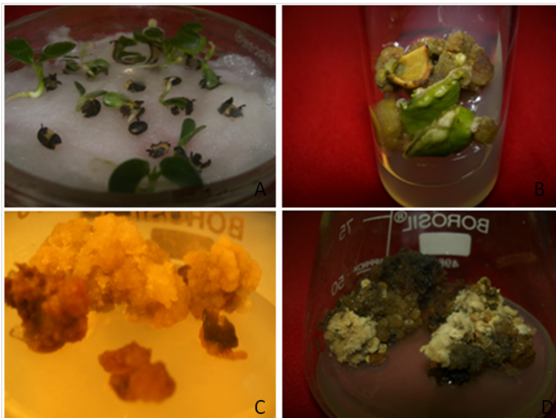

Fig.1 A-D *In vitro* callus development through cotyledonary explants of *Psoralea corylifolia* Linn. **A** seed germination after 7 days 0.86x. **B** Culture showing brownish-yellow, nodular, soft and profuse amount of callus on B5 + 10 mM 2,4-D after one month 1.89x. C Callus culture raised on B5+5µM 2, 4-D+5µM BA with 0.5% polyethylene glycol (w/v) after two month 1.75 x. **D** callus culture on B5+5µM 2, 4-D+5µM BA with 0.5% polyethylene glycol (w/v) under optimum physico-chemical conditions after 3 month 1.22x.

The plant cell cultures would be a promising alternative to avoid extinction of species and efficient source to establish cultivation for the production of biologically important secondary metabolites at commercial level (Matkowski, 2004; Mulabagal and Tsay, 2004; Tabata, 2004; Ma et al., 2006; Jain and Saxena, 2009; Karuppusamy, 2009). In present study 2,4-D alone or in combination with BA and PEG has shown the most remarkable effects on growth of callus and high productivity of psoralen from callus. In general, an increase in auxin levels, such as 2,4-D in the medium stimulates callus formation and secondary metabolites like diosgenin by *Diocorea deltoidea* (Kaul and Staba,1968), L-DOPA by *Mucuna pruriens* (Brain, 1976), ubiquinone-10 by *N. tabacum* (Ikeda et al.,1976), gymnemic acid by *Gymnema sylvestre* (Ahmed et al., 2009) and shikonin derivative by *Echium italicum L*.(Zare et al., 2010). Similarly, BA alone (Aboul-Nasr et al., 2000) or in combination with auxins (Jain and Saxena, 2009; Liu and Saxena, 2009) has promotory effect on callus formation and secondary metabolites production.

Plant expression systems offer a valuable alternative to traditional systems for the production of recombinant biopharmaceuticals (Mulabagal and Tsay, 2004). A highly efficient polyethyleneglycol (PEG)-mediated transient expression system for secreted recombinant proteins in plants has also been developed (Baur et al., 2005) and it is found that PEG changes in the transcript levels of many genes involved in sucrose catabolism and nitrogen assimilation and utilization(Stasolla et al., 2003). Now a days PEG-derived copolymers is customized for tissue-engineering applications (Tessmar and Gopferich, 2007) as it is well known as a non-metabolizable osmoticum(Chen and Dribnenki, 2004).

Psoralen estimation:
The HPLC chromatograms of five seed samples from central region of India showed different pattern (Fig.2).Among these samples, Gaziabad seeds showed best results i.e. 7655 µg/ g FW followed by Lucknow seeds, Jamia Hamdard seeds, NBPGR seeds and Kharibawli seeds sample in the order of 5719,5619, 5429 and 5267 µg/ g FW, respectively. When 7 d old cotyledons from Gaziabad seed sample were cultured on B5+5µM 2, 4-D+5µM BA with 0.5% polyethylene glycol (w/v) media up to three months under optimum physico-chemical conditions then the amount of psoralen was found to be significantly very high (16.15 mg/g FW) i.e. more than two times as compare to seeds sample (Fig.2). Under *in vitro* condition, green cotyledonary explants (7 d old) has 5298 µg/ g FW of psoralen. Calluses raised by suspension culture on B5+5 µM 2,4-D liquid media (2 month old) has 5323 µg/ g FW of psoralen. Calluses raised on semisolid media i.e.B5+5 µM 2,4-D up to two month, continuous sub culturing on same media up to 10 months and continuous sub culturing up to two years has 5442, 6068 and 7700 µg/ g FW of psoralen , respectively.

Similar to present study, several secondary metabolite products were found to be accumulated in cultured cells at a higher level than those in native plants through optimization of cultural conditions. For example, diosgenin by Dioscorea (Rokem et al., 1984), rosmarinic acid by *Colleus blumei* (Ulbrich et al., 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), berberine by *Coscinium fenestratum* (Khan et al., 2008) , azadirachtin by *Azadirachta indica* (*Sujanya* et al., 2008), flavonoid production by *Saussurea medusa* (Liu and Saxena, 2009), shikonin derivative by *Echium italicum L.*(Zare et al., 2010). Till date, no published work or combination of published works has indicated the *in vitro* production of Psoralen through cotyledonary cultures. This finding is unique which shows that cotyledon is the best source of Psoralen as compared to the whole seed. Therefore, by adopting this technology it would be possible to take up Psoralen production on a commercial/ industrial scale. The present process is globally novel and inventive. The process adopted here is simple and has no similarity to any known processes for Psoralen extraction. It can be concluded that the developed method is new, accurate, precise and specific, showing high resolution, accuracy and reproducibility.

Volume : 4 | Issue : 4 | Apr 2014 | ISSN - 2249-555X

Acknowledgments :

Author Dr Shahnaz Subhan is thankful to the Department of Science and Technology, New Delhi, India, for funding the project (Ref. No. SR/FT/L-96/2003) to her under the fast track young scientist scheme, Pharmacopoeial Laboratory for Indian Medicine, Kamla Nehru Nagar, Ghaziabad, U.P., India for providing the seed material to carry out this work successfully and Mr. M Ram Tyagi from Jamia Hamdard, Delhi to help in the HPLC.

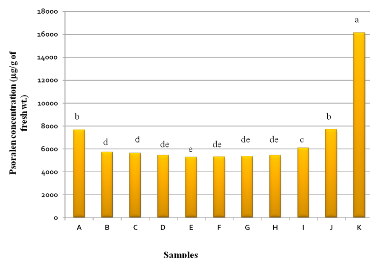

Fig.2 : Psoralen concentrations (µg/g fresh wt.) obtained from different seed samples and callus cultures of *Psoralea corylifolia* Linn.

Seed samples; A) Ghaziabad, B) Luckhnow, C) Jamia Hamdard, D) NBPGR, E) Kharibawli. Callus Cultures; F) Green cotyledon (7 day old), G) Suspension culture on B5+5µM 2, 4-D (2 months old) , H) Callus on B5+5µM 2, 4-D (2 months old), I) Callus on B5+5µM 2, 4-D (10 months old), J) Callus on B5+5µM 2, 4-D (2 years old), K) callus on B5+5µM 2, 4-D+5µM BA with 0.5% polyethylene glycol (w/v; 3 month old) under optimum physico-chemical conditions.

Values in a column followed by the same superscript are not significantly different as determined by SAS at $p \leq 0.05$.

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