



In Vitro Conservation Strategies for Sustainable Production of Secondary Metabolites in *Psoralea corylifolia* L.

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Abstract *Psorelia corylifolia* L. is a rich source of flavonoids, phenolics, antioxidants particularly psoralen with its significant biological activities. The plant products have great market demand on account of their versatile medicinal properties. Overexploitation from the wild and poor seed germination ability has enormously depleted its natural populations. Micropropagation is unconventional approach for its conservation and to fill market demand of its secondary metabolites. The present work was aimed to develop an efficient in vitro protocol for its rapid multiplication and large-scale production of secondary metabolites. Different phytohormonal combinations in Murashige and Skoog (MS) medium were used for shoot and callus induction. Maximum shoot induction (85.0%) through mature meristem culture was observed on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA. The callus induction and profuse shoot multiplication protocol was developed from leaf explants. MS medium with 1.5 mg/l BAP and 0.5 mg/l NAA was the best medium for maximum in vitro response of callus (85.0%), shooting and multiple shoots (7.0) with 8.5 cm length. A comparative analysis of psoralen content using HPLC, antioxidant

properties, total flavonoid and phenolic contents was observed in field-grown seeds and in vitro raised callus. All these parameters were significantly higher in in vitro raised callus than field-grown seeds. The present investigation can serve as a prospective guide for in vitro regeneration, consequently for conservation and sustainable production of secondary metabolites from *P. corylifolia*.

Keywords *Psoralea corylifolia* · Endangered · Callus culture · Psoralen · Secondary metabolites

Introduction

P. corylifolia L. (Fabaceae) commonly known as ‘Indian bread root’ is an erect annual herb of 30–180 cm high bearing bluish or purple flowers. It is found in both tropical and subtropical regions of the world. It abounds in wild from the plains of Central and Eastern India and is reported to be cultivated in Rajasthan and the eastern districts of Punjab [1]. The plant is conventionally used in Ayurvedic and Indian system of medicine for the treatment of skin disorders such as psoriasis, vitiligo leucoderma, leprosy, as a cardiac tonic and vasodilator [2]. Roots of the plant are useful in dental caries, and the seed has significant medicinal properties, fruits are laxative, aphrodisiac and anti-inflammatory [3]. Also, the leaves are used for the treatment of diarrhoea [4]. The plant is a rich repository of secondary metabolites which include psoralen, isopsoralen, neobavaisfloavone, bovachin, bavaislfoavone, bavachromene, psoralidin, corylifolinin, bavachinin, bavachalcone [5]. Other active constituents including neoba-vaisl-floavone, borachin, bavaislfoavooz, bavachalcone, bava-chromene psoralidin, corylifolinin, barachini psoralenoside, isopsoralenoside, coumarins and flavonoids

Significance statement The present investigation is an efficient, rapid, improved method likely to be used for industrial supply of bulk and quality plant material. It can serve a prospective guide for its future sustainable exploitation.

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[6]. Apart from a number of prenyl flavonoids and related compounds isolated from *P. corylifolia* [7], the plant is also a rich source of flavonoids, phenolics, antioxidants alkaloids, terpenoids, steroids, saponins, phenolics and amino acids. These compounds bestow the plant important medicinal properties. The plant extracts have been reported to possess antibacterial [8], antifungal [9], antioxidant [10], anti-inflammatory [11], antiparasitic [12], estrogenic [13], antitumour [14] and immunomodulatory activity [15].

Due to the versatile properties of *P. corylifolia*, its natural populations have declined very fast due to indiscriminate and illegal collections and destruction of its habitats, as a result, it is included in the endangered list of plants [16]. Low seed germination percentage, less seed viability, long gestation period and delicate field handling are some other factors which impede its commercial cultivation [17]. Moreover, being rich source of psoralen, industrial demand of this species has enormously increased. Instantaneously, psoralen-based industrial products are produced by collecting field-grown plants. Due to overutilization of whole plant parts, their natural populations are declining at an alarming rate. Hence, there is an urgent need for its conservation. Plant cell culture technology shows promise for the large-scale production of high-value secondary metabolites. This technique offers uniform secondary product synthesis by eliminating effect of unforeseen climatic conditions and diseases as observed in field-grown plants. Therefore, the development of an efficient *in vitro* propagation protocol for its conservation and for the production of secondary metabolites is an obvious reason to accomplish this purpose. The present study has been premeditated to develop a reliable protocol likely to be used for conservation and secondary metabolite production. Consequently, the present investigation is an efficient, rapid, improved method likely to be used for industrial supply of bulk and quality plant material. In this study, it was observed that *in vitro* raised callus was superior in biochemical properties than field-grown seeds. Therefore, present study deals with the state of art and future challenges of plant cell culture technology for psoralen, total flavonoid, and phenolic content production in *P. corylifolia*.

Material and Methods

Explant Collection

The plants were identified at CSIR IIIM canal road Jammu. Voucher specimen (Acronym RRLH, Accession no. 22949) was prepared and submitted to Janaki Ammal herbarium, CSIR IIIM canal road Jammu (J and K).

Explant and Medium Preparation

Different explants, i.e. nodal segments, axillary buds and leaf segments of 5–10 cm length excised from the mother plants maintained in nursery beds were taken for shoot and callus induction experiments. The explants were washed under running tap water and then washed thoroughly in sterile double distilled water. The chemical treatment of the explants for complete sterilization was given in 1.0% Bavistin (Carbendazim Powder BASF India Limited) a broad spectrum fungicide for 10 min followed by 5.0% (v/v) Teepol (Qualigens Fine Chemicals, India), a liquid detergent for 5 min by continuous shaking method. The chemically treated explants were washed in sterile double distilled water for 4 to 5 times. Further, surface sterilization treatment was conducted in a laminar airflow chamber. The explants were then surface-sterilized by immersing in a freshly prepared solution of 3.0% (w/v) NaOCl (Qualigens Fine Chemicals, India) for 4 to 5 min under laminar flow. Finally, the explants were washed 3 to 5 times with sterile double distilled water for 5 min to remove all traces of sterilizing agents.

The stock solutions of MS medium (macronutrients, micronutrients, iron and organic) were prepared. The medium was supplemented with various growth regulators and other growth adjuncts as per requirement. The thermostable growth regulators were added to the medium before making up the total final volume with distilled water.

The media was prepared in culture tubes (25 × 150 mm, Borosil) and prior to autoclaving pH of the media was adjusted to 5.8 using 0.1 N. NaOH (sodium hydroxide) or 0.1 N. HCl (hydrogen chloride) with a pH metre (Lab India, India) and was solidified by adding 1% agar (HiMedia Lab. Ltd., India) and sterilized by autoclaving at 15 lb pressure per square inch, 121 °C temperature for 15 min. The surface-sterilized explants were then inoculated on MS medium supplemented with various concentrations of BAP, Kn, 2,4-D, NAA, IAA and IBA shown in (Tables 1–6) either singly or in various combinations for shoot induction, callus induction, shoot regeneration, multiplication, callus production and root formation. The cultures were incubated at 25 ± 2 °C with relative humidity of 55 ± 5% and exposed to photocycle of 2500 Lux intensity for 16 h. Visual observations like shoot induction, number of days taken for bud break, percentage of bud break, callus induction, morphology of callus, length of shoots regenerated per explants and secondary metabolite accumulation and length of roots regenerated per shoot were recorded after 3 weeks as shown in (Tables 1–6). After micropropagation of *P. corylifolia* via direct or indirect organogenesis, the well-developed rooted plantlets were taken out from the culture

Table 1 Effect of growth regulators either alone or in combination in MS medium on shoot initiation of *Psoralea corylifolia*

MS + Auxin/ cytokinin (mg/L)	%age of bud break	No. of days required	Mean No. of shoots ± SE	Mean shoot length in cm.± SE*
0.5 BAP	40.3	30	2.0 ± 0.3	3.0 ± 0.9
1.0 BAP	65.5	25	4.0 ± 0.21	5.0 ± 0.23
0.5 KN	35.1	35	1.7 ± 0.8	2.4 ± 0.1
1.0 KN	50.0	30	2.7 ± 0.8	3.5 ± 0.9
0.5 BAP + 0.5 IBA	45.0	30	2.5 ± 0.1	3.7 ± 0.9
0.5 BAP + 0.5 NAA	60.9	25	3.0 ± 0.4	4.4 ± 0.5
0.5 KN + 0.5 IBA	40.0	30	2.1 ± 0.1	3.2 ± 0.1
0.5 KN + 0.5NAA	42.5	30	2.5 ± 0.8	3.4 ± 0.1
1.5 BAP + 0.5 NAA	85.0	21	5.5 ± 0.8	7.0 ± 0.2
1.0KN + 0.5 NAA	50.5	25	3.0 ± 0.9	4.0 ± 0.1

*SE (LSD = Least Significant Difference)

medium flasks and washed thoroughly with running tap water to remove all traces of medium attached to the roots for hardening. Finally, the tissue culture-raised plantlets were planted in hardened pots containing mixture of soil, sand and farmyard in the ratio of (1:2:1) for acclimatization and were maintained in green house.

Initiation and Mass Production of Callus

For the production of secondary metabolites, the mass callus cultures were produced. Callus was initiated by using sterile cotyledonary leaves as explants. Explants of size 1–2 mm were excised and inoculated on MS medium containing different concentrations of cytokinins like 0.2–1.0 mg/l BAP, 0.2–1.0 mg/l Kn and auxins like 0.5–2.0 mg/l 2,4-D. The cultures were maintained in dark for fifteen to twenty days. For mass production of callus fifteen to twenty days old callus obtained from cotyledonary leaves were further transferred either to the same or to different medium supplemented with higher concentration of growth hormones like 0.5–2.0 mg/l 2, 4-D either alone or in combination with cytokinins like 0.2–1.0 mg/l BAP and 0.2–1.0 mg/l Kn.

Quantification of Psoralen Content in Field Seeds/ Callus by HPLC Analysis

To prepare extracts for analysis, 100 gm dried powder of callus and 100 gm dried powder of field seeds were separately soaked in methanol (500 ml in each sample) for 24 h. After 24 h, the methanolic extracts were then filtered through filter paper, and the supernatant was transferred into another pre-weighed round bottom flasks. The residue left was re-extracted twice with 500 ml methanol by same

procedure as was performed in the first step after 24 h. Thereafter, the residue left was discarded, and the supernatant was pooled, filtered and evaporated to dryness in a rotary evaporator at 40°C. The extracts thus obtained were analyzed by using high-performance liquid chromatography (HPLC). Extracts of field seeds and callus were transferred to 10 ml standard flask. Volume was made up to the mark with acetonitrile: water (40:60), sonicated for 10 min. It was filtered with 0.22µ filter to obtain sample stock solution. The sample solution was further diluted and was made upto 100 ng/ml concentrations. Then, it was filtered with 0.22µ filter. Sample solution was analysed at wavelength of 237 nm of 10 µl injection volume. Stock solution of psoralen (1 mg/ ml) was prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at -20 °C. Quantification was carried out using 5 levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 20–100 ng/ ml. Each concentration of standard was filtered through a 0.22 µm nylon membrane filter (Millipore, the USA) before HPLC. An accurately weighed quantity of psoralen (10 mg) was transferred to a 10 ml volumetric flask, dissolved and diluted to the mark with acetonitrile: acetic acid (40:60) to obtain standard stock solution of 1000 µg/ml. Psoralen content was quantified using HPLC as described earlier [18].

Quantification of Antioxidants, Flavonoid Content and Phenolic Content

Estimation of antioxidants, flavonoids and phenolics was carried out in field seeds / callus of *P. corylifolia*. For this, crude extracts were prepared from 100 mg dried sample of field seeds and from 100 mg dried sample of callus. Shade

dried plant tissues were powdered and extracted thrice with methanol at 30 °C (with sonication) for 3 h. The crude extracts thus obtained were analyzed using spectrophotometer (Nano Drop 2000c, Thermo Fisher Scientific, the USA). Measurement of radical-scavenging activity of field seeds and callus was carried out according to the method described by [19]. Ascorbic acid was used as positive control, and % inhibition was determined according to the following equation:

$$\text{Percent (\%) Inhibition} = \frac{[(ADPPH - AS)/ADPPH] \times 100.}$$

where (AS) is the absorption of the solution when the sample extract was added at a particular concentration and ADPPH is the absorbance of the DPPH solution. Three biological and technical experimental replicates were taken for the assay. IC₅₀ values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical, a lower IC₅₀ value corresponded to a higher antioxidant activity of sample [19].

The flavonoid content was quantified using spectrophotometer as described earlier (Kaur et al. 2013). Briefly, dried crude extracts dissolved in 500 µl of distilled water were mixed with 30 µl of 5% NaNO₂ solution followed by 5 min of incubation at room temperature. After this, 300 µl of 10% AlCl₃.H₂O solution was added, and the sample was further incubated for 6 min. Finally, 200 µl of 1 M NaOH and 200 µl of distilled water were added to the sample and absorbance read at 510 nm. Total flavonoid content using quercetin as standard (10–100 µg; R₂ = 0.998) was calculated. The results were expressed as mg quercetin equivalent (mg QAE) per gram dry weight of plant material.

The phenolic content of methanolic extracts was determined as per the method adopted by (Prior et al. 2005) in both field-grown seeds as well as in vitro regenerated callus. Methanolic extract of 500 µg in 100 µl was mixed with 100 µl of 1 N Folin–Ciocalteu reagent. Following incubation for 5 min, 200 µl of 20% Na₂CO₃ was added. Absorbance at 730 nm was recorded in plate reader after 10 min, and the concentration of phenolic compounds was calculated using standard curve of gallic acid (500–5000 ng; R₂ = 0.967). The results were expressed as mg gallic acid equivalent (mg GAE) per gram dry weight of plant material.

Data and Statistical Analysis

Visual observations were recorded frequently in terms of number of cultures responding for axillary and apical shoot proliferation, shoot development, number of shoots per explant, average length of regenerated shoots, number of roots per shoot and average length of root. All the

experiments were conducted with a minimum of twenty explants. All assays were repeated at least thrice. The experimental data were statistically analyzed by one-way ANOVA (Analysis of variance) using the DMRT (Duncan's Multiple Range Test) ($P < 0.05$) and were presented as the mean \pm SE (standard error).

Results and Discussion

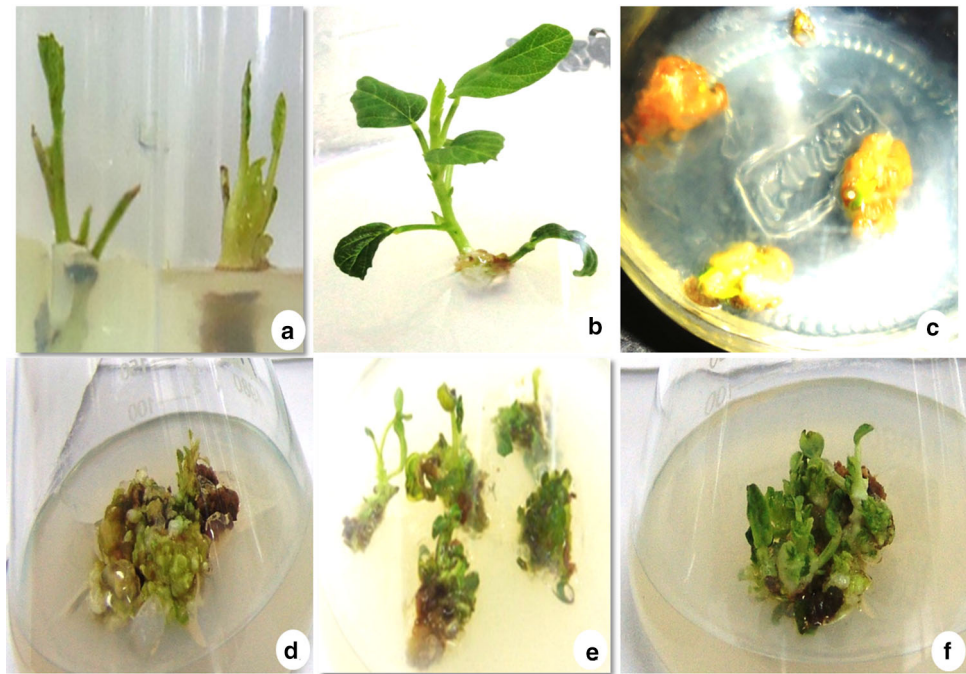
Establishment of Cultures

The shoot induction through nodal explants on MS media supplemented with BAP and Kn along with auxins, i.e. IBA IAA and NAA revealed a significant variation in terms of bud break and number of shoots induced per explant. Shoots initiated from mature meristem explants showed excellent response in every medium tried in the experiment. The young axillary buds of the newly sprouted branches were more responsive. Maximum shoot induction (85.0%) was observed in the media supplemented with 1.5 mg/l BAP in combination with 0.5 mg/l NAA in terms of number of shoot buds 5.5 ± 0.8 with the length of 7.0 ± 0.2 cm within 21 days Fig. 1.(a, b) and Table 1. However, minimum shoot induction (40.0%) was observed on media containing 0.5 mg/l Kn with 0.5 mg/l IBA with number of shoots 2.1 ± 0.1 with the length of 3.2 ± 0.1 cm within 30 days. Hence, for maximum (85.0%) bud break and shoot initiation from young axillary and apical bud, MS media 1.0 mg/l BAP with 0.5 mg/l NAA phytohormonal combination proved the most effective followed by 1.0 mg/l BAP (65.5%) alone. It was also observed that addition of auxin 0.5 mg/l IBA does not show any effect on shoot initiation (Table. 1). The shoot clusters obtained were found to retain their vigour and health. The initiated shoots were further transferred to fresh medium for multiplication experiment. Previously, the highest shoot regeneration (75%) has been achieved on MS medium with 12 µM/l BAP and 10.0 µM/l NAA and 15.0 µM/l Kn [20]. Many other investigations have also reported similar findings in *P. corylifolia* [21]. In their study, MS medium with 5.0–10.0 mg/l BAP and 0.5 mg/l NAA has been found optimal for shoot induction. As per wide the literature survey, current study is efficient in terms of use of lowest concentration of plant growth regulators for shoot induction on MS media within minimum time period.

Callus Induction and Shoot Multiplication

In current study, several experiments by using leaf segments for callus induction were carried out to study the effect of different concentrations and combinations of 2,

Fig. 1 Effect of different phytohormones on different developmental stages: axillary bud as explant (a), direct plant regeneration (b) callusing (c), morphogenesis of callus and multiple shoot induction (d–f)



4-D (0.5–2.0 mg/l) either alone or with BAP (0.5 mg/l) and Kn (0.5 mg/l) (Table. 2). It was observed that in most of the media combinations, explants either callused or differentiated into shoots. Maximum callus (80.0%) was induced from the cut ends of explants on MS medium supplemented with 1.0 mg/l of 2, 4-D and 0.5 mg/l Kn within 25 days. The callus was greenish, friable and embryogenic in nature. On the other hands, the callus produced in 2, 4-D alone was greenish brown and compact. However, the combination of 0.5 mg/l 2, 4-D and 0.5 mg/l BAP resulted in reduced callus formation (40.8%) after 30 days. The differentiated callus from the previous experiments was subsequently removed and transferred to

new medium combinations for shoot regeneration (Fig. 1 c-f). The present study is in corroboration with the previous investigations on *Rauvolfia serpentina* [22] and *Phyllanthus amarus* [23]. Different types and concentrations of auxins and cytokinins in the medium and subsequently their possible interactions or cross-talk between exogenous and endogenous plant growth regulators have a marked effect on the in vitro culture responses. Although 100% of cultures showed regeneration response in medium containing cytokinin /auxin, but best shoot regeneration (85.0%) was observed on MS medium supplemented with 1.5 mg/l BAP with 0.5 mg/l NAA. In this media combination, about 7.0 ± 0.2 numbers of microshoots were

Table 2 Effect of growth regulators either alone or in combination in MS medium on callus induction in *Psoralea corylifolia*

MS + Auxin/cytokinin (mg/L)	%age of callus induction	No. of days required	Morphology of callus
0.5 2, 4-D	45.0	30	Dark green
1.0 2, 4-D	75.5	30	Green brown compact
2.0 2, 4-D	50.3	35	Dark Brown and compact
3.0 2, 4-D	40.2	40	Green
0.5 2, 4-D + 0.5 BAP	40.8	30	Greenish
1.0 2, 4-D + 0.5 BAP	33.5	30	Dark green
2.0 2, 4-D + 0.5 BAP	30.5	35	Greenish yellow compact
1.0 2, 4-D + 0.5 KN	45.4	25	Greenish fragile
1.5 2, 4-D + 0.5 KN	80.0	30	Greenish brown and compact
2.0 2, 4-D + 0.5 KN	30.5	40	Brownish green soft

observed with the length of 8.5 ± 0.8 cm within 25 days (Table.3). Also, it was observed that the callus enlarged to adventitious shoot-buds directly from the cut ends of the explants, but few shoot-buds were differentiated from callus derived from leaf segments when the medium was supplemented with 2.0 mg/l of Kn and 0.5 mg/l of IAA. In this combination, numbers of shoots 4.9 ± 0.7 with the length of 6.0 ± 0.7 cm were observed within 30 days. On the other hands, only 40.0% of shoot buds were differentiated from callus on the medium supplemented with 1.0 mg/l of Kn with 0.5 mg/l of NAA. In this combination, the numbers of shoots 2.0 ± 0.1 with the length of 3.0 ± 0.1 cm were achieved within 30 days. Hence, cytokinins particularly 1.0 mg/l BAP with 0.5 mg/l NAA combination operated as trigger for shoot induction from the callus and was most effective among all the phytohormonal combinations tried Table 4. Similarly, the synergistic effect of BAP with NAA was observed in *Glinus lotoides* [24], *Casuarina cunninghamiana* [25] and *Saussurea obvallata*, [26]. However, in *P. corylifolia*, higher number of shoots (6.15) were obtained on 14 μ M/l BAP with 10.0 μ M/l NAA and 10.0 μ M/l Kn from leaf-derived callus [21].

In order to get profuse and rapid shoot multiplication, medium supplemented with cytokinin and auxin combinations was also used. Auxins, like IBA and NAA, were supplemented with varied concentration of BAP and Kn. Nevertheless, addition of auxins to the medium did not show any significant positive effect on multiplication fold and shoot elongation. A prolonged incubation of 6 weeks instead of the usual 5 weeks culture period resulted in the significant increase in shoot formation and elongation Fig. 2 (a-d). It was observed that every medium was responsive and the cultures showed highest percentage

(85.0%) of shoot formation with an average of 12.0 ± 0.1 adventitious shoots, having length of 14.0 ± 0.8 cm directly from the regenerated shoots, without any intermittent callus formation on medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA within 20 days (Table 4).

However, only a few shoots have been observed on Kn in the concentration of (0.5 mg/l) with IBA and NAA. An average of 50.0- 60.0% of shoot multiplications was reported over a period of 30 days with number of shoots 4.3 ± 0.1 with the length of 5.8 ± 0.9 cm. Therefore, from all concentrations 1.0 mg/l BAP with 0.5 mg/l NAA produced the most desirable results both in terms of multiplication fold and cluster of perfective elongated shoots. The results obtained in the current study are parallel with the earlier reports on *P. corylifolia* for highest rate of shoot multiplication [27]. In contrast, the synergistic effect of BAP with NAA has been observed in the current study which has previously been reported from *chrysanthemum* [28].

Rooting of In Vitro Regenerated Shoots

The induction of roots has been observed in every medium tried. Medium with root-inducing growth regulators at the concentration of 0.5 to 1.5 mg/l IAA, 0.5 to 1.5 mg/l NAA and 0.5 to 2.0 mg/l IBA was tested from in vitro raised shoots. Maximum (80.0%) root induction was achieved directly from the base of the shoots on medium supplemented with 1.0 mg/l IBA of average length 8.0 ± 0.9 cm within 25 days Fig. 2d. In contrast, by increasing the concentration of IBA, root regeneration decreased considerably. Root initiation 60.5% was observed on medium supplemented with 1.5 mg/l of IBA having length of 5.0 ± 0.9 cm within 25 days (Table. 5).

Table 3 Effect of growth regulators either alone or in combination in MS medium on shoot regeneration from callus of *Psoralea corylifolia*

MS + Auxin/cytokinin (mg/L)	%age of shoot regeneration	No of days required	Mean No. of shoots produced \pm SE	Mean shoot length in cm \pm SE*
0.5-BAP	40.9	30	2.8 ± 0.5	3.0 ± 1.0
0.5-KN	35.5	25	2.0 ± 0.1	2.7 ± 0.14
1.0-BAP	68.5	25	5.1 ± 0.0	6.5 ± 0.5
1.0-KN	50.2	30	4.0 ± 0.11	5.5 ± 0.11
0.5-BAP + 0.5-IBA	55.4	30	4.6 ± 0.1	5.8 ± 0.8
0.5-KN + 0.5-IBA	45.5	35	3.7 ± 0.3	4.5 ± 0.9
1.5-BAP + 0.5-IAA	85.0	25	7.0 ± 0.2	8.5 ± 0.8
1.0- KN + 0.5-NAA	40.0	30	2.0 ± 0.1	3.0 ± 0.1
2.0- BAP + 0.5- IAA	65.0	30	4.9 ± 0.7	6.0 ± 0.7
2.0- KN + 0.5- IAA	42.0	35	2.6 ± 0.1	3.1 ± 0.1

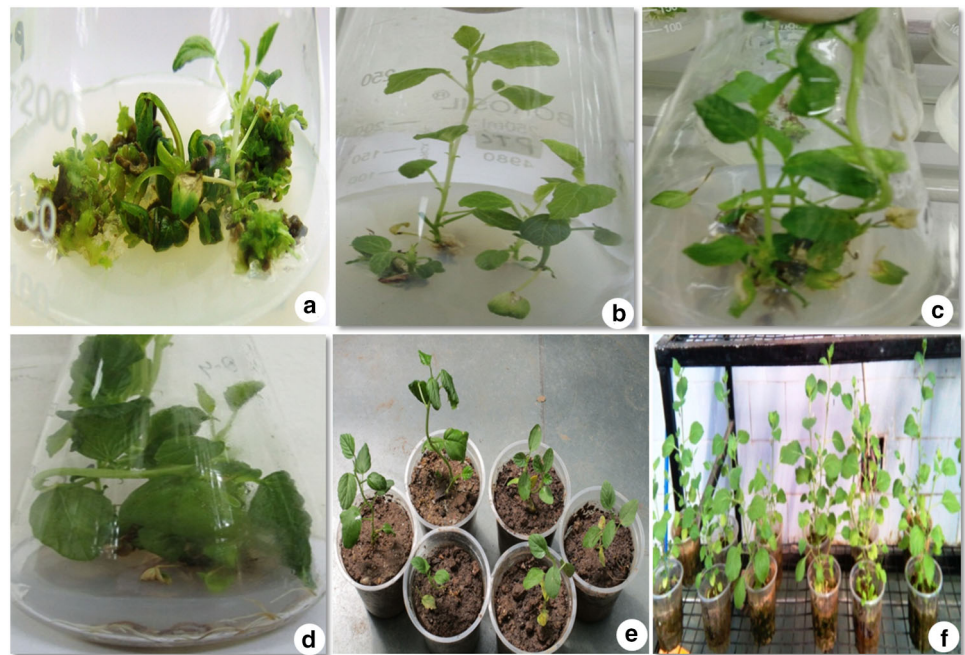
*SE (LSD = Least Significant Difference)

Table 4 Effect of growth regulators either alone or in combination in MS medium on shoot multiplication from induced shoots of *Psoralea corylifolia*

MS + Auxin/cytokinin (mg/L)	%age of shoot multiplication	No of days required	Mean No. of shoots produced \pm SE*	Mean shoot length in cm. \pm SE*
0.5 BAP	40.0	20	3.0 \pm 1.2	3.9 \pm 0.02
0.5 KN	30.5	25	1.0 \pm 0.52	2.0 \pm 0.3
1.0 BAP	50.5	25	4.0 \pm 0.6	5.2 \pm 0.01
1.0 KN	35.0	30	2.0 \pm 0.1	3.0 \pm 0.5
1.0 BAP + 0.5 IBA	55.0	30	4.5 \pm 0.6	5.2 \pm 0.7
1.0 KN + 0.5 IBA	45.0	30	3.5 \pm 0.6	4.4 \pm 0.1
1.0 BAP + 0.5 NAA	85.0	20	12.0 \pm 0.1	14.0 \pm 0.8
1.5 KN + 0.5 NAA	60.0	25	5.0 \pm 0.7	7.0 \pm 0.2
2.0 BAP + 0.5 NAA	70.9	25	7.0 \pm 0.2	9.5 \pm 0.6
2.0 KN + 0.5 NAA	50.5	30	4.3 \pm 0.1	5.8 \pm 0.9

*SE (LSD = Least Significant Difference)

Fig. 2 Effect of different phytohormones on, multiple shooting from callus (**a, b**) efficient rooting in rooting medium (**c, d**) hardening and acclimatization of in vitro raised plants transferred in root trainers containing potted mixture for four weeks in mist chamber (**e**) and full grown hardened plants in open environment (**f**)



Minimum (55%) root induction was observed on higher concentrations (2.0 mg/l) of IBA. Roots were induced along with extensive callusing at the basal end of shoots which hindered its further growth. The roots formed were very slender and thin in the medium containing 1.0 mg/l NAA and IAA and showed only 40.0–45.5% of root induction after 25 days with the length of 3.7 ± 0.6 cm, while as 1.0 mg/l IAA and 1.0 mg/l NAA failed to develop any root system. Among the various concentration and combinations full strength MS basal medium supplemented with 1.0 mg/l IBA showed best root induction with healthy roots within minimum time period as shown in (Table 5).

Similar results were reported in *P. corylifolia* by [21]. Superiority of IBA for root induction has been reported earlier in many other plant species such as in *G. lotoides* (L.) [24], *Eclopta alba* L. [28], *Chrysanthemum* [29].

Hardening and Field Transfer

The transfer of plants from the culture flasks to the soil requires a careful, stepwise procedure. After 15 to 20 days of culture on rooting media, the rooted plantlets were transplanted to pots for hardening prior to their final transfer to soil. Rooted plantlets were taken out of the

Table 5 Effect of growth regulators in MS medium on root induction of *Psoralea corylifolia*

Media composition (mg/l)	% age of root induction	No. of days required	Mean root length in cm. \pm SE*
MS + 0.5 IAA	45.0	30	3.5 \pm 0.7
MS + 0.5 IBA	50.0	25	4.0 \pm 0.3
MS + 0.5 NAA	40.0	20	3.0 \pm 0.3
MS + 1.0 IAA	40.5	20	3.0 \pm 0.9
MS + 1.0 IBA	80.0	25	8.0 \pm 0.9
MS + 1.0 NAA	45.5	25	3.7 \pm 0.6
MS + 1.5 IAA	55.5	25	4.5 \pm 0.2
MS + 1.5 IBA	60.5	25	5.0- \pm 0.9
MS + 1.5 NAA	50.0	30	3.2 \pm 0.2
MS + 2.0 IBA	55.0	25	4.4 \pm 0.8

*SE (LSD = Least Significant Difference)

culture bottles with the help of forceps and washed thoroughly with water to remove any remaining of the medium. A minimal survival rate of 40–50% was recorded during the months of May, June, July and August. However, the plants taken out after September showed a substantial increase in survival percentage. This may be due to the ample moisture content in the atmosphere in this season. All hardened plants survived on transfer to pots in greenhouse containing soil, sand and farmyard in ratio of (1:2:1) mixture Fig. 2 e, f.

Secondary Metabolite Production

Twenty-five to thirty days old induced callus was sub-cultured on MS medium with different combinations of BAP, Kn and 2, 4 D to produce sufficient callus for secondary metabolite production. The highest percentage of callus induction (85.0%) was observed on medium containing 1.5 mg/l 2, 4-D with 0.5 mg/l KN within 30 days (Fig. 3) followed by 75% on medium containing 1.0 mg/L 2,4-D with 1.0 mg/L KN within 35 days, and the callus formed was light yellow greenish in colour. The yellowish brown callus so obtained showed the accumulation of phytochemicals (Table. 6) (Fig. 3 a–c). The chemical production from the 4–6-week-old callus cultured on different nutrient media was analyzed by using HPLC. However, this medium could be a suitable medium and noble approach to produce maximum amount of callus within short time period. Results indicated that the accumulation of phytochemicals in *P. corylifolia* callus can be effectively modified with auxins and cytokinin supplemented into culture media. The concentration of plant growth regulators was reported to be crucial factors in secondary metabolite production. The type and

concentration of auxin, cytokinin or auxin/cytokinin ratio alter dramatically both the growth and medicinally important compound produced in plant cell culture. The 2, 4-D has been shown to inhibit the production of secondary metabolites in a large number of plants. Elimination of 2, 4-D or replacement of 2, 4-D by Kn has been shown to enhance the production of anthocyanins in callus cultures of *Daucus carota* [23]. It is recommended that development of callus through in vitro method offers the possibility of obtaining desirable medicinal compounds as well as ensuring sustainable conservation and rational utilization of biodiversity.

The amount of total psoralen was determined by HPLC method. Psoralen was used as a standard compound, and the psoralen was expressed as ng/ml using the standard curve. The HPLC analysis was performed on the extracts of field seeds and callus for the determination of principle compound psoralen (Fig. 4). The HPLC chromatogram of seed extract and callus depicted R value at 6.6 min, by using solvent system acetonitrile: water (40:60). The peaks which could be identified in the chromatogram were psoralen. The psoralen content in callus was found to be significantly higher 60.58 ng/ml than the methanolic extract of field seeds 40.55 ng/l. Hence, the HPLC chromatogram exhibited that quantity of psoralen present in callus extract was more compared to extract of field seeds (Fig. 5). Production of useful secondary metabolites from plant cell culture has been regarded as a potential solution to the supply constraint. Cell cultures have been found to produce higher levels of secondary metabolites than the differentiated mother plant itself. It is therefore, imperative to develop ways for increasing the bioactive compounds and separate the phytochemical constituents and develop methods for its large-scale in vitro production [20]. The

Fig. 3 Effect of different phytohormones on efficient callusing consequently used for the extraction of bioactive compounds through chemical screening. Callus regeneration (a) multiplication of callus (b) hardened callus (c) and dried callus for chemical investigation (d)

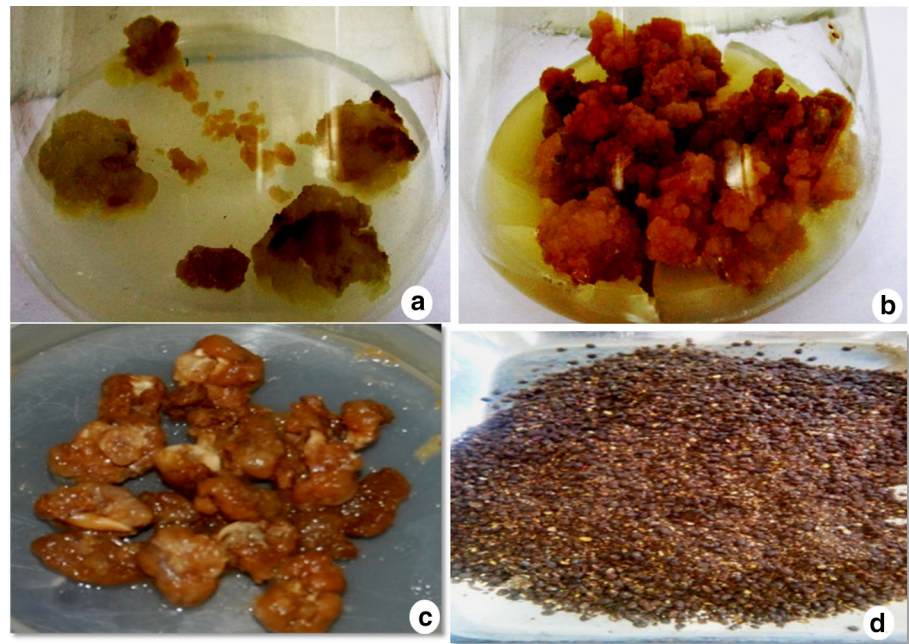


Table 6 Effect of growth regulators either alone or in combination in MS medium on callus induction for secondary metabolite production in *Psoralea corylifolia*

Medium code	MS + auxin/cytokinin (mg/L)	Percentage callus induction	Days Taken	Morphology of callus	Callus Weight (mg)	
					Fresh weight	Dry weight
P.S.M.1	0.5-2, 4-D	40.5	30	Greenish yellow	1690	140
P.S.M.2	1.0- 2,4-D	30.0	35	Light yellow green and soft	1850	148
P.S.M.3	0.5-2, 4-D + 0.2-BAP	30.3	35	Yellow compact	1750	145
P.S.M.4	0.5-2, 4-D + 0.2-KN	43.2	30	Yellow green	1600	140
P.S.M.5	1.0-2, 4-D + 0.5- BAP	65.8	30	Dark Green embryogenic	1600	141
P.S.M.6	1.5-2, 4-D + 0.5-KN	85.0	30	Yellow brown	2100	158
P.S.M.7	1.0-2, 4-D + 1.0-BAP	35.5	35	White compact	1500	131
P.S.M.8	1.0-2, 4-D + 1.0-KN	75.0	35	Light Yellow greenish callus	1590	139
P.S.M.9	2.0-2, 4-D + 1.0-BAP	45.4	30	Yellow soft	1700	148
P.S.M.10	2.0-2, 4-D + 1.0-KN	70.0	35	Creamish soft callus	1500	112

current validated method can prove to be simple, fast, accurate, precise and sensitive and thus can be used for routine analysis of psoralen in *P. corylifolia*.

Quantification of Antioxidant Activity (DPPH Radical-Scavenging Activity)

DPPH method is a simple, rapid, sensitive and reproducible assay used for measuring the antioxidant activity of plant extracts. In the DPPH assay, an antioxidant scavenges the

free radicals and is used to measure the capacity of extracts to scavenge the stable radical DPPH formed in solution by donation of hydrogen atom or an electron [29]. The IC₅₀ value for methanolic extract of callus was found to be 303.32 ± 34.08 and that of field seeds was 148.47 ± 0.12 . Hence, extract prepared from field seeds of *P. corylifolia* shows less antioxidant activity as compared to the extract of callus (Fig. 5b).

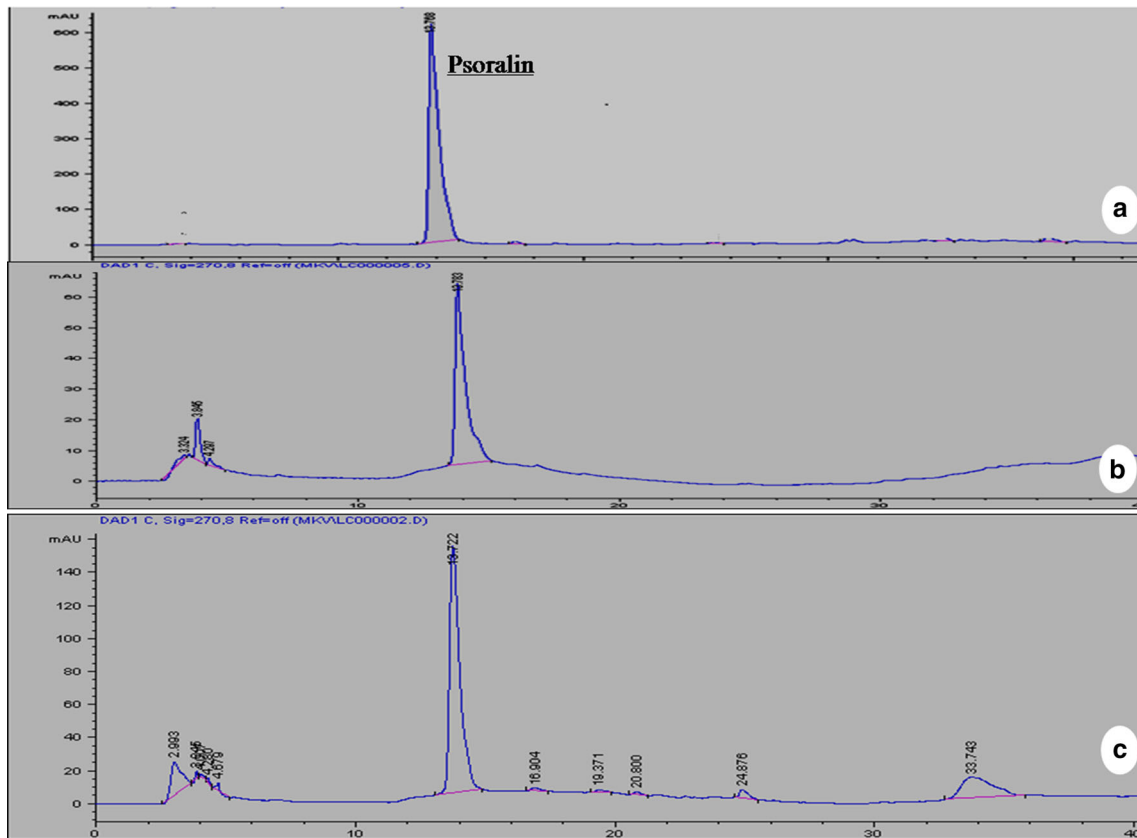


Fig. 4 Quantification of psoralen content by HPLC analysis, chromatogram of pure compound as standard psoralen (a), field seed extract (b) and in vitro regenerated callus (c). (Concentration = ng/ml)

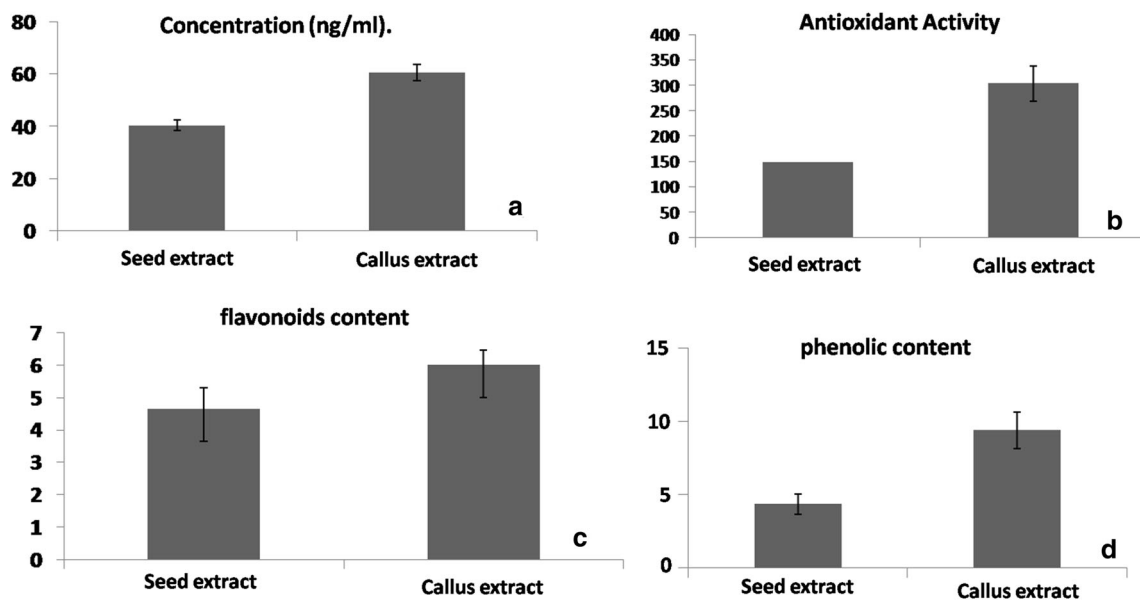


Fig. 5 Measurement of different parameters from field seed extract and in vitro regenerated callus: psoralen content ng/ml (a) antioxidant activity using DPPH radical-scavenging assay (b) total flavonoid content (c) and total phenolics (d)

Quantification of Flavonoid and Phenolic Content

Flavonoids significantly contribute to the antioxidant property to the plant. Total flavonoid content in callus of *P. corylifolia* was found to be significantly higher in comparison to field seeds, i.e. 6.0 ± 0.45 and 4.63 ± 0.66 , respectively (Fig. 5c). Also, the total phenolic content in callus was found to be significantly higher than the field seeds, i.e. 9.4 ± 1.24 and 4.35 ± 0.70 , respectively (Fig. 5d). Enhanced production of flavonoids has been reported from in vitro regenerated shoots and roots of *P. corylifolia* as compared to field-grown plants [30]. Production of secondary metabolites from the easy grown callus under in vitro conditions can act as a recurrent source for the conservation of the species in its wild. This is due to satisfying the elevated demand of the important metabolites from the species.

Conclusion

In the present study, a fruitful indirect organogenesis protocol was developed for *P. corylifolia* through callus formation, shoot regeneration and successful regeneration of new plantlets. We demonstrate that the protocol developed can be used for conservation of elite germplasm and true to type mass propagation of this herb of immense pharmaceutical relevance. This is highly advantageous for a range of further biotechnological applications and will also help in the production of improved plants. Total psoralen content of in vitro raised callus of *P. corylifolia* was significantly higher than the field-grown seeds. Also, the total flavonoid content, antioxidant potential and total phenolic content in the callus of *P. corylifolia* were found to be significantly higher than the field seeds. At present, the research into psoralen production through cell, tissue culture is in its very initial state and there is long way to go before the establishment of an economical viable process. To develop feasible efficient culture technology for psoralen production, optimization of all physiological, environmental and bioengineering aspects of plant tissue culture is highly warranted.

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Author Contributions TAW conceived and designed the experiments; NGN performed bulk of the experiments as a part of her PhD programme; Support for chemical analyses was provided by ZAK; Data were analyzed by T.A.W.; ZAK, T.A.W. and NGN wrote the paper. All authors read, reviewed and approved the manuscript.

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