ज्ञान-विज्ञान विमुक्तये

UNIVERSITY GRANTS COMMISSION

Bahadur Shah ZafarMarg

New Delhi - 110 002.

Submission of Final Report for UGC – Major Research Project 2012 – 2015

Project entitled

Isolation, Characterization and enhancing the production of Psoralen from *Psoralea* corylifolia L. through in vitro culture

(UGC Letter No. F. No. 41-393/2012/(SR)/Dt.16.07.2012 & F. No. 41-393/2012/(SR), Dt. 19.06.2015)

By

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Annexure - VIII

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI - 110 002.

Final Report (1st July 2012 to 31st December 2015) of the work done on the Major Research Project entitled "Isolation, Characterization and enhancing the production of Psoralen from *Psoralea corylifolia* L. through *in vitro* culture"

1.	Project report No. 1st/2nd/3rd/Final	:	**Final**
2.	UGC Reference No.	:	**F. No. 41-393/2012/(SR)/Dt.16.07.2012** **F. No. 41-393/2012/(SR), Dt. 19.06.2015**
3.	Period of report	:	**1st July 2012 to 31th December 2015**
4.	Title of research project	:	**Isolation, Characterization and Enhancing the production of Psoralen from *Psoralea corylifolia* L. through *in vitro* culture**
5.	(a) Name of the Principal Investigator	:	**Prof. N. JAYABALAN**
	(b) Department	:	**Plant Science**
	(c) University/College where work has Progresse	:	**Bharathidasan University**
6.	Effective date of starting of the project	:	**01.07.2012**
7.	Grant approved and expenditure Incurred during the period of the report		
	a. Total amount approved Rs.	:	**Rs. 11, 99,800/-**
	b. Revised Amount Rs.	:	**Rs. 11,53,933/-**
	c. Total grant received	:	**Rs. 10, 76,720/-**
	d. Total expenditure Rs.	:	**Rs. 11,51,012/-**
	e. Report of the work done	:	See **Annexure –I**

8. Brief objective of the project

- **To standardize the protocol for *in vitro* regeneration of *Psoralea corylifolia* L. by using different explants.**
- **Development of mass root culture system through bubble type Bioreactors. The roots will be induced from leaves, axillary bud and roots by direct and callus mediated organogenesis to find out the efficient system in terms of secondary metabolite production.**
- **To standardize optimum production bioactive compounds through verifying different duration of *Agrobacterium* - mediated transformation protocol for hairy roots growth with Methyl jasmonate and Salicylic acid added as elicitor.**
- **To assess the Psoralen and Isopsoralen through TLC, GC-MS and HPLC.**

ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication)

9. PUBLICATIONS

I. Published articles

1. Siva, G., Sivakumar, S., Premkumar, G., Baskaran, P., Senthilkumar, T., and **Jayabalan, N.** (2014). Enhanced seed germination of *Psoralea corylifolia* L. by heat treatment. World Journal of Agricultural Research 2(4): 151-154.
2. Siva, G., Sivakumar, S., Premkumar, G., Senthil Kumar, T., and **Jayabalan, N.** (2015). Enhanced production of psoralen through elicitors treatment in adventitious root culture of *Psoralea corylifolia*L. International Journal of Pharmacy and Pharmaceutical Sciences, 7 (1), 146-149.
3. Siva, G., Sivakumar, S., Prem Kumar, G., Vigneswaran, M., Vinoth, S., MuthamilSelvan, A., ParveezAhamed, A., Manivannan, K., Rajeshkumar, R., Thajuddin, N., Senthil Kumar, T., and **Jayabalan, N.** (2015). Optimization of elicitation condition with Jasmonic Acid, characterization and antimicrobialactivity of Psoralen from direct regenerated plants of *Psoralea corylifolia*L. Biocatalysis and Agricultural Biotechnology, 4(4), 624-631.
4. Siva, G., Sivakumar, S., Prem Kumar, G., Vigneswaran, M., Vinoth, S., Arunachalam, S., Elango, B., Senthil Kumar, T., and **Jayabalan, N.** (2015). Multiple shoot production from nodal explant and FTIR analysis of *in vitro*regenerated plants of *Psoralea corylifolia*L. Pharmaceutical and Biological Evaluations, 2(4), 105-109.

II. Conference Proceedings

1. Siva, G., Sivakumar, S., Prem Kumar, G., Vigneswaran, M., Senthil Kumar, T., and **Jayabalan,N**. Enhanced production of psoralen in adventitious root culture of Psoralea corylifolia through abiotic elicitors treatment. XXXVIII All India Conference of The Indian Botanical Society and National Symposium on Emerging Trends in Plant Sciences, Department of Botany, University of Rajasthan, Jaipur, October 2015.

2. Siva, G., Sivakumar, S., Vinoth, S., Prem kumar, G., Vigneswaran, M., Senthil Kumar, T., and **Jayabalan, N.** Adventitious root culture and enhancement of Psoralen production through elicitors treatment in *Psoralea corylifolia* L. International Conference on Trends in Plant Systematics, Bharathidasan University; October 2014.

3. Siva, G., and **Jayabalan, N**. An efficient protocol for direct regeneration of *Psoralea corylifolia* L. from hypocotyl explant. National seminar on Recent Trends in Plant Science, Department of Plant Biology and Plant Biotechnology, Sivakasi, September 2014.

4. Siva, G., and **Jayabalan, N**. HPLC analysis of psoralen from *Psoralea corylifolia* L. through indirect regeneration. Recent Trends in Bioprospecting of Plants, Department of Plant Science, Bharathidasan University, Tiruchirappalli, March 2014.

5. Siva, G., and **Jayabalan, N**. HPLC analysis of Psoralen from *Psoralea Corylifolia* L. through direct regeneration. National Symposium on Himalayan Biodiversity: Prospects and Challenges, North-Eastern Hill University, Shillong, March 2014.

iii. Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons

The progress of the project has been achieved towards objectives.

iv. Please indicate the difficulties, if any, experienced in implementing the project

-Nil

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet.

Not Applicable

vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.

See Annexure - I & II

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any

(a) Manpower trained - 1

(b) Ph. D. awarded - 1 (Thesis Submitted)

(c) Number of Publications - 4

(d) Impact if any -

Signature of the Principal Investigator

Signature of the Registrar

Annexure - IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI–110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project	: **Isolation, Characterization and enhancing the production of Psoralen from *Psoralea corylifolia* L. through *invitro* culture**
2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR	: **Prof. N. JAYABALAN**
3. NAME AND ADDRESS OF THE INSTITUTION	: **Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu.**
4. UGC APPROVAL LETTER NO. AND DATE	: **F. No. 41-393/2012 (SR)/Dt.16.07.2012**
	F. No. 41-393/2012/(SR), Dt. 19.06.2015
5. DATE OF IMPLEMENTATION	: **01.07.2012**
6. TENURE OF THE PROJECT	: **3 years**
7. TOTAL GRANT ALLOCATED	: **Rs.11, 99,800/-**
8. REVISED GRANT ALLOCATED	: **Rs.11,53,933/-**
8. TOTAL GRANT RECEIVED	: **Rs. 10, 76,720/-**
9. FINAL EXPENDITURE	: **Rs. 11,51,012/-**
10. TITLE OF THE PROJECT	: **Isolation, Characterization and enhancing the production of Psoralen from *Psoralea corylifolia* L. through *invitro* culture**

11. OBJECTIVES OF THE PROJECT :

- **To standardize the protocol for *in vitro* regeneration of *Psoralea corylifolia* L. by using different explants.**
- **Development of mass root culture system through bubble type Bioreactors. The roots will be induced from leaves, axillary bud and roots by direct and callus mediated organogenesis to find out the efficient system in terms of secondary metabolite production.**
- **To standardize optimum production bioactive compounds through verifying different duration of *Agrobacterium* - mediated transformation protocol for hairy roots growth with Methyl jasmonate and Salicylic acid added as elicitor.**
- **To assess the Psoralen and Isopsoralen through UV, NMR, FTIR, and HPLC.**

12. WHETHER OBJECTIVES WERE ACHIEVED :

Isolation, Characterization and enhancing the production of Psoralen from *Psoralea corylifolia* L. through *invitro* culture. In this present study using different elicitors and production of Psoralen was achieved.

13. ACHIEVEMENTS FROM THE PROJECT : **Published four articles**

14. DETAILED SUMMARY OF THE FINDINGS : **See annexure - II**

15. CONTRIBUTION TO THE SOCIETY (GIVE DETAILS) : **See annexure - III**

16. WHETHER ANY Ph.D. ENROLLED/ PRODUCED OUT OF THE PROJECT : One (Thesis Submitted)

17. NO. OF PUBLICATIONS OUT OF THE PROJECT (PLEASE ATTACH) : 4

PRINCIPAL INVESTIGATOR **REGISTRAR/PRINCIPAL**

Annexure I

Project Title:

Isolation, characterization and enhancing the production of Psoralen from *Psoralea corylifolia* L. through *in vitro* culture

1. Introduction

Psoralea corylifolia Linn. is an endangered medicinally important and distributed in the tropical region of the world (Jain, 1994). From time to time the fruits, seeds and roots of *P. corylifolia* have been examined and a large number of compounds have been reported earlier (Bajwa *et al.,* 1972). The major compounds of this plant are psoralen, angelicin, psoralone, isopsoralone, bavachin and daidzein (Bouque *et al.,* 1998). Psoralen is a pharmaceutical interested compound because of their photosensitizing, photobiological and phototherapeutic properties which is used for the photochemotherapy of vitiligo and skin diseases such as psoriasis, mycosis fungoides and eczema (Frank *et al.,* 1998; Yones *et al.,* 2005). The plant is also used in indigenous medicine as laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic in febrile conditions, (Rastogi and Mehrotra, 1990) for the treatment of leucoderma, leprosy and inflammatory diseases of the skin as a paste or ointment (Anonymous, 1988; Orient longman, 1996). Many Indian pharmaceutical industries have used seeds of *P. corylifolia* as a raw material to produce medicines and Ayurvedic skin care soaps (Baskaran and Jayabalan, 2007). Propagation of *P. corylifolia* through seed is unreliable due to its poor germination rate and the high mortality of young seedlings under natural conditions (Faisal and Anis, 2006). Hence, high frequency rapid mass propagation remains a major bottleneck. An efficient *in vitro* seed germination technique is therefore required for *in situ* and *ex situ* conservation and clonal propagation of *P. corylifolia.*

1.1 Taxonomic position of *P. corylifolia* L.

According to Bentham and Hookers Classification (1862-1883)

Class	:	Dicotyledons
Sub-class	:	Ploypetalae
Series	:	Calyciflorae
Order	:	Rosales
Family	:	Leguminosae (fabaceae)
Sub family	:	Papilionoideae

Genus : *Psoralea*

Species : *corylifolia*

The genus *Psoralea* includes 130 species, distributed in the tropic and sub tropic of both hemispheres (Willis, 1966). *Psoralea corylifolia* Linn. is an important medicinal plant used in Folk, Siddha and Ayurvedic system of medicine. It is an endangered and rare herbaceous medicinal plant and distributed in the tropical region of the world [Jain, 1994]. Globally the species is distributed in Pakistan, India, Sri Lanka, Myanmar, China and Arabia. Within India, it is seen along roadsides and waste places of the tropical regions.

It has some usage in Traditional Chinese Medicine under the name Bu Ghu Zhi and has been used in Ayurveda with the name Kushtanashini. Traditional records exist for the usage of *Psoralea* in asthma, cough, nephritis, vitiligo and calvities (baldness). It is also commonly referred to as Karpokarisi in Tamil and Babchi in Hindi (Baskaran and Jayabalan; Kushboo *et al.,* 2010). Most parts of the plant (roots, leaves, seeds and seeds) appear to be used (Kushboo *et al.,* 2010) with the seeds being the most commonly used source. The seed-oil is used externally for the treatment of leucoderma, psoriasis and leprosy in Indian folk medicine. The plant has been used in Ayurvedic medicinal system as a cardiac tonic, vasodilator and pigmentor. It is widely used in Chinese medicine to treat a variety of diseases and possesses antitumor, antibacterial, cytotoxic and antihelmenthic properties. Thermally sensitive bakuchiol, psoralen and isosporalen, the major components present in the seed possess high medicinal values (Manohar and Udaya Sankar, 2012).

1.4 Importance of psoralen

Of the several bioactive compounds present in *P. corylifolia*, psoralen is one of the important furanocoumarin abundantly available in this plant which is also widely employed as an anticancerous agent against leukemia and other cancer lines (Latha *et al.,* 2000; Oliveira *et al.,* 2006; Xin *et al.,* 2010).

Furocoumarins are natural plant metabolites characterized by a furane moiety fused to benzopyran-2-one. Furocoumarins intercalate in double-stranded DNA, and psoralens are known to cross-link pyrimidine bases under irradiation by [2+ 2] cycloaddition via their 3, 4- and 2, 3-double bonds (Kanne *et al.,* 1982). The position of the urane substitution distinguishes two large groups of compounds, the linear (psoralens) and the angular furocoumarins (angelicin and derivatives) (Croteau *et al.,* 2000). Psoralen, an important

furocoumarin is known for its photosensitizing and phototoxic effects and has been used in photo chemotherapy of skin disorders (psoriasis, vitiligo, and mycosis). Due to the complex bioactivity of furocoumarins, its biosynthesis has received continuous attention. Knowledge of the biosynthetic pathway of psoralen may enable to influence its formation in a direct way by metabolic pathway engineering. The biosynthetic pathways to the linear furanocoumarin (Psoralen) involved enzymes (and their cofactors) which are as follows 1. DMAPP-umbelliferone dimethylallyl transferase 2. Marmesin synthase (O_2, cytochrome P450, NADPH), 3. Psoralen synthase (O_2, Cyt, P450, NADPH) (Zhao *et al.,* 2005). Growing commercial interest in the production of Psoralen for the development of pharmacological agents posed the interest to investigate the *in vitro* production of active principles in *P. corylifolia* cultures.

2. Materials and methods

2.1.1 Surface sterilization and inoculation of seeds

Fresh and mature seeds of *P. corylifolia* were collected from Experimental Garden, Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The seeds were subjected to heat treatment (Siva *et al.,* 2014). Seeds were washed thoroughly in tap water for 10 min, followed by soaking in soap solution (2% Teepol - commercial soap solution) for 5 min and then seeds were kept under running tap water for 30 min. The seeds were then disinfected with 70% ethanol for 45 s and rinsed with double distilled water for 3 times, followed by 0.1% (w/v) aqueous mercuric chloride treatment exposure for 5 min. The surface-disinfected seeds were inoculated into *in vitro* seed germination medium.

2.1.2 Medium preparation for *in vitro* seed germination

Medium for *in vitro* seed germination was prepared in culture tube (Borosil, India) containing growth regulator-free Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg B_5 vitamins (Gamborg *et al.,* 1968) with 3% (w/v) sucrose (Hi-media, India) and 0.8% agar (Hi-media, India). The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving. Approximately, 10 mL media was dispensed in each culture tube and autoclaved at 1.06 kg cm^{-2} at 121ºC for 15 min.

The sterilized seeds were inoculated and maintained in dark condition for 48 h at 25±2°C and then transferred to 16 h light and 8 h dark photoperiod condition with light supplied by cool white fluorescent lamps (Philips, India) at an intensity of 80 µ mol m^{-2} s^{-1}.

2.1.3 Medium preparation for multiple shoot induction

Medium for multiple shoot induction was prepared in culture tube (Borosil, India) Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg B_5 vitamins (Gamborg *et al.*, 1968) with 3% (w/v) sucrose and 0.8% agar (Hi-media, India). Plant growth regulators for multiple shoot induction and elongation along with Jasmonic Acid were added to the medium and pH was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving. Approximately, 10 mL media was dispensed in each culture tube and autoclaved at 1.06 kg cm^{-2} at 121ºC for 15 min.

2.1.4 Direct organogenesis

Shoot tip explants (2.5 cm in length) were isolated from 15 days old aseptically grown seedlings and inoculated on MSB_5 medium supplemented with 0.5 – 3.0 mg/L TDZ, 0.5-3.0 mg/L BAP, 0.5 – 3.0 mg/L Kin for multiple shoot induction and shoot elongation.

Nodal explants (2.0 cm in length) were isolated from 15 days old aseptically grown seedlings and inoculated on MSB_5 medium supplemented with Kin (0.3 – 0.8 mg/L), BAP (0.4 – 0.9 mg/L) and GA_3, (0.3 – 0.8 mg/L) for multiple shoot induction as well as shoot elongation.

Data on number of explants responding for shoot bud induction, average number of shoot bud per explants and number of elongated shoot were recorded after four weeks of culture.

2.1.5 Indirect organogenesis

Leaf and cotyledon explants were isolated from 15 days old sterile *in vitro* grown seedlings. Leaf explants (0.5 cm) were inoculated on MSB_5 medium supplemented with 0.5 – 3.0 mg/L 2,4-D and Kin for organogenic callus induction. Well prolific callus was transferred to multiple shoot induction medium supplemented with 2,4-D, TDZ and Kin (0.5-2.5 mg/L).

Cotyledon explants (0.5 cm) were inoculated on MSB_5 medium supplemented with 0.5 – 3.0 mg/L BAP, IBA and 2,4-D (0.5 - 2.5 mg/L) for organogenic callus induction. Well prolific callus was transferred to multiple shoot induction medium supplemented with 2iP and BAP (0.5-2.5 mg/L).

Data on number of explants responding for callus induction, average number of shoot bud per explant and number of elongated shoot were recorded after four weeks of culture.

2.1.6 Rooting and acclimatization of plantlets

Elongated shoots (4 cm) were excised and transferred to MS medium and B_5 vitamin supplemented with 0.2 – 1.2 mg/L IAA, 0.2 – 1.2 mg/L NAA and 0.2 – 1.2 mg/L IBA. The rooted plants were removed from the culture tubes, washed free of agar with sterile distilled water and transferred to paper cups with sterile potting mixture of soilrite and vermiculite in the ratio of 1:1 and maintained in plant growth chamber (Sanyo, Japan) with 70% relative humidity, temperature 25°C and 14h light/10h dark photoperiod.

2.2 Elicitor treatment

2.2.1 Adventitious Root culture

Leaf explants were collected from 20 day-old *in vitro* raised seedlings. The trimmed leaf explants (0.5 cm) were inoculated in mMS medium containing 3% sucrose, NAA (0.5-2.5 mg/L), IAA (0.5-2.5 mg/L) and IBA (0.5-2.5 mg/L). The cultures were maintained in dark condition for 48 h at 25±2 °C and then under 16 h photoperiod condition with a light intensity of 80 µ mol m^{-2} s^{-1}. After root induction from leaf explants, the roots were transferred into liquid root culture medium (250 mL conical flask) containing 3% sucrose, NAA (0.5-2.5 mg/L), IAA (0.5-2.5 mg/L) and IBA (0.5-2.5 mg/L).

2.2.2 Preparation of elicitors

Elicitors were prepared by dissolving in their respective solvents. Methyl jasmonate (MeJ) and Jasmonic acid (JA) were dissolved in 99.9% ethanol. Salicylic acid (SA) and silver nitrate ($AgNO_3$) was dissolved sterile distilled water. Dissolved elicitors were filter sterilized using 0.22 µ membrane filter and stored in -20ºC until further use.

2.2.3 Optimization of elicitor concentrations

The prepared elicitors were used at different concentrations viz. MeJ (10, 20, 30 and 40 µM/L), SA, JA and $AgNO_3$ (50, 100, 150 200 and 250 µM/L). The culture was maintained in an orbital shaker at 120 rpm/min. The 28^{th} day of liquid root culture was exposed for 8 h contact time with the elicitors for the production of psoralen.

After 8 h of elicitation treatment, the root samples were collected from liquid root culture medium, blot dried to remove medium constituents and fresh weight of each root sample was determined using electronic balance (Shimadzu, Japan). Growth Index of the root culture was calculated by the following formulae (Dhanya *et al.,* 2014).

$$\text{Growth index} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

2.2.4 Preparation of root extracts

After elicitation treatment, the root samples were harvested from the liquid root culture medium, dried and ground to fine powder. The powder was extracted with 10 mL methanol using sonication for 30 min. The extracted material was then subjected to dry at 50 ºC for 1 week and then the sample was again dissolved in 5 mL methanol and centrifuged at 8000 rpm for 25 min. The supernatant was then filtered through 0.22 µ membrane filter and it was subjected to HPLC analysis.

2.2.5 HPLC analysis for estimation of psoralen content

The methanolic extract of elicitor treated root samples was analyzed by HPLC on a (Waters, Australia) reverse phase C-18 (5.0 µm particle size) 250 × 4.6 mm column. The mobile phase was methanol/water (50:50 *v/v*) mixture supplied at the rate of 0.8 mL/min. The absorbance range was set as 210-400 nm. The analytical HPLC was performed using Empower 2 software following isocratic method. The psoralen peak was detected by monitoring absorbance at 254 nm using a PDA detector (Baskaran and Jayabalan, 2008). The peak of psoralen, in methanolic extract was compared with peak of psoralen standard (Sigma Aldrich, India).

2.2.6 Estimation of psoralen from elicitor treated roots:

Estimation of psoralen content in elicitor treated root samples were compared with standard and calculated by the following formula (Siva *et al.,* 2015).

$$\text{Sample concentration} = \frac{\text{Sample area}}{\text{Mean STD Area}} \times \frac{\text{STD weight}}{\text{STD Dilution}} \times \frac{\text{Sample Dilution}}{\text{Sample Weight}}$$

2.3 *Agrobacterium rhizogenes*-mediated transformation

2.3.1 Explant preparation

Surface sterilization and *in vitro* seed germination of *P. corylifolia* seeds were done as mentioned in previous chapter. Leaf explants were collected from 15 days-old *in vitro* seedlings and used for initiation of hairy root culture.

2.3.2 Hairy root culture

A single colony of *Agrobacterium rhizogenes* strain A4 (pRiA4) was inoculated into liquid Luria-Bertani (LB) medium and grown to OD_{600} = 0.6 at 28 ºC in an orbital shaker at 180 rpm for 24 h. The bacterial cells were pelleted by centrifugation at 5000 rpm for 10 min and resuspended in half strength liquid mMS medium containing 15 g/L sucrose. Leaf explants were immersed in the bacterial inoculums for 30 min. The explants were blotted on sterile filter paper (Whatman No.1) remove excess bacterial inoculum. LB liquid medium without bacteria was applied to the explants as a control. The infected leaf explants were co-cultured on mMS medium under dark condition at 25±2 ºC (Binns and Thomashow, 1988)

A minimum of 20 explants were used for each experiment. All the explants were cultured on sterilized petriplates comprising solid [0.8 % (w/v) agar] MS (Murashige and Skoog, 1962) medium supplemented with sucrose 30 g/L and myo-inositol 100 mg/L (Himedia Mumbai, India) The medium was gelled with 0.8% (w/v) agar (Hi-media Mumbai, India). The pH was adjusted to 5.8 ± 0.2. The medium was autoclaved under 1.06 kg/cm2 pressure at 121 ºC for 15 min.

2.3.3 Elimination of *A. rhizogenes* from hairy roots

The co-cultured leaf explants were washed with half-strength mMS liquid medium and then transferred to fresh mMS medium containing 250 mg/L Cefotaxime. The subculture was done with mMS medium + 250 mg/L Cefotaxime for every 15 days. The hairy roots were cultured in LB medium to check the growth of *Agrobacterium* for axenic hairy root growth .

Cefotaxime was omitted after three successive subcultures at 15 days intervals. For suspension culture, bacterial-free hairy roots were transferred to 250 mL Erlenmeyer flask containing 15 g/L sucrose for hairy root establishment. The data were collected after 4 weeks from solid and suspension cultures.

The results were expressed in percentage transformation frequency.

$$\%\ \text{transformation frequency} = \frac{\text{number of explants inducing hairy roots}}{\text{total number of explants infected with } A.rhizogenes} \times 100$$

All the experiments were repeated thrice with 20 explants per treatment in each experiment and the results were expressed as mean + SE. Statistical analysis was performed according to DMRT ANOVA (SPSS version 17).

2.3.4 Bioreactor culture

Bioreactor culture was performed in a 5 L bubble type bioreactor (BTB) (Lark Innovative, India) containing 3 L of half-strength liquid mMS medium containing 15 g/L sucrose. Hairy roots (15 g/L FW) were inoculated into BTB. All cultures were kept in the dark at 25±2 ºC for 5 weeks.

To determine the optimal aeration volume for both root biomass and psoralen compound production, various aeration volumes of 2-10 LPH (gradually increased at a 1-week interval) were tested. After 5 weeks, growth parameters such as fresh weight, dry weight, growth index and psoralen content were assessed.

2.3.5 Measurement of Growth Parameters

Fresh weight estimation. Fresh weight of root cultures was determined after blotting on Whatman No. 1 filter paper. Fresh weight was recorded as g/L. Fresh weight was measured by harvesting hairy roots after a culture period of 15 d.

Dry weight estimation. Dry weights were determined after drying the samples in a hot air oven at 60 ºC for 24 h. Dry weight was recorded as g/L. Dry weight was measured by harvesting hairy roots after a culture period of 15 d.

Growth Index of the root culture was calculated by the following formulae (Dhanya *et al.*, 2014).

$$\text{Growth index} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

2.3.6 Extraction and analysis of psoralen content

Hairy roots which showed best growth index were taken for HPLC analysis to determine psoralen content. Hairy root were shade dried and soaked in methanol for 24 h under dark condition and then homogenized using mortar and pestle. After evaporation, the extracted sample was dissolved in methanol (Sigma, HPLC grade). The mixture was centrifuged for 15 min at 8,000 rpm at room temperature. The supernatant was then filtered

through 0.2 µm membrane filter and subjected to HPLC analysis. Then the root extract was analyzed using NMR.

2.3.7 Analytical HPLC

The methanolic extract of hairy roots was analyzed by HPLC on a (Waters, Australia) reverse phase C-18 (5.0 µm particle size) 250 × 4.6 mm column. The mobile phase was methanol/water (50:50 *v/v*) mixture supplied at the rate of 0.8 mL/min, using a sample temperature of 25 °C during the analysis. The absorbance range was set as 210-400 nm.The analytical HPLC was performed using Empower 2 software following isocratic method. The psoralen was detected by monitoring absorbance at 254 nm using a PDA detector (Baskaran and Jayabalan, 2008). The peak of psoralen, in the methanolic extract was compared with Psoralen standard (Sigma Aldrich, India). The amount of psoralen was calculated by the following formulae (Siva *et al.*, 2015).

$$\text{Sample concentration} = \frac{\text{Sample area}}{\text{Mean STD Area}} \times \frac{\text{STD weight}}{\text{STD Dilution}} \times \frac{\text{Sample Dilution}}{\text{Sample Weight}}$$

2.3.8 Preparative HPLC

The methanolic extract of hairy roots was subjected to preparative HPLC on a (Waters, Australia) reverse phase C-18 (10 µm particle size) 250 × 10 mm column. The sample was injected at 5 mL volume. The mobile phase methanol/water (50:50 *v/v*) mixture was supplied at the rate of 3.5 mL/min. The absorbance range was set as 210-400 nm. The preparative HPLC was performed using Empower 2 software following isocratic method. The psoralen peak was eluted at the retention time of 20.172 using a Waters Fraction collector III.

2.3.9 Fourier Infrared (FTIR) spectroscopy analysis

In Fourier transform infrared (FTIR) analysis, the FTIR spectrum of the HPLC purified sample was recorded on a PerkinElmer 1600 instrument in the range 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.3.10 Nuclear Magnetic Resonance Spectroscopic Analysis

^{1}H nuclear magnetic resonance (NMR) spectra were recorded to confirm the structure of psoralen in HPLC purified sample at 67 °C in DMSO (Dimethyl sulfoxide) using a Bruker spectrometer (operating at 400.13 MHz) and were assigned by comparison of chemical shifts. Chemical shifts were reported as d values relative to tetramethylsilane (TMS) as internal reference.

3. Results

3.1 *In vitro* regeneration

Multiple shoot production from shoot tip explants of *P. corylifolia* was achieved with MSB5 medium containing different concentrations of plant growth regulators. MSB_5 medium supplemented with 2.0 mg/L BAP was gave best result for shoot multiplication with a mean of 16.3± 0.24 shoots per explant with 82.2 % of response TDZ induced only 9.4 ± 0.42 shoots per explant with 61.3 % of response but KIN improved shoots with 13.1 ± 0.18 shoots per explant and 70.3 % of response (Fig.3), (Table 1).

Nodal explants resulted multiple shoot production (44.5% and 19.1± shoots per explant) with a combination of KIN (0.3 mg/L) and BAP (0.4 mg/L). GA_3 and BAP at concentration of 0.4 and 0.5 mg/L showed 9.1% of response and mean number of 17.9 shoots per explant (Table 3, Fig 4).

MSB_5 medium supplemented with 0.8 mg/L IAA was the most effective for root induction with mean of 18.6 ± 0.34 roots per explant with 82.6 % of response (Table 2). Different concentrations of auxins (IAA, NAA and IBA) were used to induce root formation. NAA at 0.5 mg/mL was found to be suitable for highest root induction with 72.2 % of response with mean number of 15.2 roots per shoot (Table 4).

Shoot tip explants showed better regeneration efficiency than nodal explant. This observation is contrary to the earlier report made by Anis and Faisal (2005). MS medium without growth regulator did not initiate shoot bud differentiation. Significant differences were observed in number of shoots per explant among different concentrations and combinations of growth regulators.

Direct multiple shoot induction is the useful means of production of plantlet from young plants with a lower risk of genetic instability than by the other regeneration routes, hence direct regeneration without intervening callus phase, is a more reliable method for clonal propagation (Thorpe, 1993). Adventitious shoot regeneration is also most preferred regeneration pathway if *Agrobacterium*-mediated gene transfer is to be achieved (Kantia and Kothari, 2002). The results of the present study clearly demonstrates that the high frequency shoot bud and plantlet regeneration can be achieved from shoot tip and nodal explants of *Psoralea corylifolia*.

Multiple shoot induction from shoot tip and nodal explants was highly influenced by combinations of plant growth regulators. In the present study, shoot tip explants cultured on cytokinins and auxin supplemented medium produced shoots after 2 week of culture. The number of shoot buds increased significantly after 4 week while no shoot regeneration was observed in control. Cytokinin has already shown significant effects on shoot regeneration in *P. corylifolia* from different explants (Jeyakumar and Jayabalan, 2002; Faisal and Anis, 2006; Shinde *et al.,* 2009). The synergistic positive effect of BAP and Kin was quite evident in the present study. The combination of two cytokinins has also been found to be more effective than alone (Chalupa, 1987; Nielsen *et al.,* 1995).

Different concentrations of auxins and cytokinins were used for organogenic callus production. Callus was initiated from the cut ends of the explants. The callus rating and morphogenic response were assessed after 2 weeks of culture. Both leaf and cotyledon explants produced callus at all hormone treatments, while no callus was observed in control. With both explants, 2,4-D was found to be better in respect to the initiation and subsequent proliferation of callus than other hormones tested. Among different concentrations, 1.5 mg/L 2,4-D produced green compact callus from leaf explant and 1 mg/L 2,4-D produced green compact callus after 2 weeks of culture. The calli differentiated into shoot bud in the treatments after two weeks of culture on multiple shoot induction medium. Medium supplemented with Kin at 2 mg/L induced 9.9 mean number of shoots from leaf derived callus whereas 2iP resulted 17.6 number of shoots from cotyledon derived callus. In the present study, the callus growth was fast in the first 2 weeks culture, later slowed down with deep brown and finally declined. This was due to phenolic exudation and therefore, two week-old green compact nodular callus suitable for rapid shoot regeneration in *P. corylifolia*.

Induction of green compact callus was observed from leaf explant on MSB_5 medium containing 1.5 mg/L 2,4-D with 84.8% of response (Table 5). MSB_5 medium supplemented with 2 mg/L Kin showed best callus production with 68.7% of response and mean number of 9.9 shoots/callus (Fig 5, Table 6). Best callus induction from cotyledon explant was achieved on MS medium containing 1.0 mg/L 2,4-D with 53.2% of response (Table 8). Multiple shoot induction from cotyledon derived callus was found to be higher on MSB_5 medium containing 2iP 1.5 mg/L with 98.9% of response and 17.6 mean number of shoots per callus (Fig 6, Table 9). Multiple shoots were subcultured on the same medium for proliferation and

elongation. The regenerated shoots were excised and transferred to MS basal medium supplemented with single auxins such as IAA, IBA or NAA for rhizogenesis.

Well rooted plantlets were successfully hardened in paper cups containing sand:vermiculite:soilrite (2:1:1). Rooting of the elongated shoots was the best obtained with 0.4 mg/L NAA with 94.3% of root induction and mean number of 17.9 roots/shoot (Table 3.10).

Table 1: Effect of MSB_5 medium supplemented with cytokinins on multiple shoot induction and proliferation from shoot tip explant

Plant growth regulators (mg/L)	Multiple Shoot Induction	
	Percentage of response	Mean number of shoots/shoot tip explant
TDZ		
0.5	56.1 ± 0.45^{m}	8.6 ± 0.31^{m}
1.0	48.7 ± 0.48^{p}	7.2 ± 0.18^{p}
1.5	67.4 ± 0.25^{f}	12.8 ± 0.21^{f}
2.0	$\mathbf{61.3 \pm 0.45^{jk}}$	$\mathbf{9.4 \pm 0.42^{jk}}$
2.5	43.5 ± 0.78^{q}	6.1 ± 0.36^{q}
3.0	35.9 ± 0.89^{r}	5.8 ± 0.28^{r}
KIN		
0.5	61.2 ± 0.56^{k}	9.1 ± 0.11^{k}
1.0	49.5 ± 0.52^{o}	7.3 ± 0.13^{o}
1.5	$\mathbf{70.3 \pm 0.81^{c}}$	$\mathbf{13.1 \pm 0.18^{c}}$
2.0	62.7 ± 0.48^{h}	9.6 ± 0.21^{h}
2.5	56.9 ± 0.47^{l}	8.9 ± 0.14^{l}
3.0	50.1 ± 0.61^{n}	7.4 ± 0.16^{n}
BAP		
0.5	65.2 ± 0.63^{g}	11.5 ± 0.27^{g}
1.0	68.5 ± 0.54^{d}	12.9 ± 0.16^{d}
1.5	75.7 ± 0.43^{b}	15.6 ± 0.21^{b}
2.0	$\mathbf{82.2 \pm 0.72^{a}}$	$\mathbf{19.1 \pm 0.24^{a}}$
2.5	67.8 ± 0.37^{ef}	12.3 ± 0.16^{ef}
3.0	61.3 ± 0.65^{ij}	9.1 ± 0.12^{ij}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p<0.05$). Best results are indicated in bold. Average number of shoot buds per explant and number of elongated shoots were recorded after four weeks of culture on MSB_5 medium supplemented with cytokinins.

Table 2: Effect of MSB_5 medium supplemented with auxins on root induction in elongated shoots

Plant growth regulators (mg/L)	Rooting	
	Percentage of response	Mean number of roots/shoot
IAA		
0.2	64.5 ± 0.47^{c}	15.1 ± 0.12^{c}
0.4	68.5 ± 0.38^{b}	16.8 ± 0.31^{b}
0.6	60.3 ± 0.59^{e}	14.3 ± 0.13^{e}
0.8	$\mathbf{82.6 \pm 0.55^{a}}$	$\mathbf{18.6 \pm 0.34^{a}}$
1.0	54.2 ± 0.74^{f}	13.9 ± 0.46^{f}
1.2	46.7 ± 0.51^{jk}	12.7 ± 0.19^{jk}
NAA		
0.2	47.2 ± 0.53^{j}	12.9 ± 0.41^{j}
0.4	$\mathbf{61.3 \pm 0.45^{d}}$	$\mathbf{14.4 \pm 0.32^{d}}$
0.6	53.2 ± 0.64^{g}	13.8 ± 0.81^{g}
0.8	46.6 ± 0.51^{k}	12.3 ± 0.73^{k}
1.0	42.8 ± 0.21^{n}	10.6 ± 0.35^{n}
1.2	31.2 ± 0.1^{o}	8.3 ± 0.21^{o}
IBA		
0.2	44.1 ± 0.42^{l}	11.1 ± 0.42^{l}
0.4	43.2 ± 0.63^{m}	10.7 ± 0.31^{m}
0.6	$\mathbf{53.0 \pm 0.41^{h}}$	$\mathbf{13.7 \pm 0.43^{h}}$
0.8	47.1 ± 0.31^{ij}	12.9 ± 0.19^{ij}
1.0	27.4 ± 0.53^{p}	4.3 ± 0.29^{p}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p<0.05$). Best results are indicated in bold. Mean number of roots per shoot were recorded after four weeks of culture on MSB_5 medium supplemented with auxins.

Table 3: Effect of cytokinins on multiple shoot induction from nodal explant of *Psoralea corylifolia* L

Plant growth regulators (mg/L)		Shoot formation	
		Percentage of response	Mean number of shoots/explant
KIN + BAP			
0.3	**0.4**	**44.5 ± 0.36 [a]**	**16.3 ± 0.11[a]**
0.4	0.5	16.5 ± 0.22[j]	13.3 ± 0.13[j]
0.5	0.6	28.5 ± 0.51[g]	14.1 ± 0.18[g]
0.6	0.7	32.5 ± 0.28[e]	16.4 ± 0.21 [e]
0.7	0.8	15.2 ± 0.47[k]	13.9 ± 0.14[k]
0.8	0.9	35.1 ± 0.61[cd]	17.4 ± 0.16[cd]
GA_3 + BAP			
0.3	0.4	26.2± 0. 53[h]	10.5 ± 0.27[h]
0.4	**0.5**	**39.1±0.64[b]**	**17.9 ± 0.16[b]**
0.5	0.6	35.5±0.43[c]	15.6 ± 0.21[c]
0.6	0.7	22.1±0.72[i]	9.3 ± 0.24[i]
0.7	0.8	14.3±0.37[l]	7.3 ± 0.16[l]
0.8	0.9	31.0± 0. 65[f]	14.1 ± 0.12[f]

Data were collected after 20 days of culture in MSB_5 medium supplemented with cytokinins and GA_3; Best results are indicated in bold letters. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of shoot buds per explant and number of elongated shoots were recorded after four weeks of culture on MSB_5 medium supplemented with cytokinins.

Table 4: Effect of different concentrations of auxins on root induction of elongated shoots

Plant growth regulators (mg/L)	Rooting	
	Percentage of response	Mean number of roots/shoot
IAA		
0.3	**43.5 ± 0.47 ^{g}**	**15.1 ± 0.02^{g}**
0.4	20.5 ± 0.39 ^{m}	09.8 ± 0.91^{m}
0.5	12.3 ± 0.09 ^{n}	8.3 ± 0.11d^{n}
0.6	11.6 ± 0.05^{o}	7.6 ± 0.10 ^{o}
0.7	11.0 ± 0.45 ^{p}	7.0 ± 0.43 ^{p}
0.8	10.4 ± 0.11 ^{q}	06.4 ± 0.09 ^{q}
NAA		
0.3	33.2 ± 0.53^{j}	11.0 ± 0.11 ^{j}
0.4	65.5 ± 0.65^{b}	13.4 ± 0.42 ^{b}
0.5	**72.2± 0.56^{a}**	**15.2 ± 0.91 ^{a}**
0.6	41.8± 0.31^{c}	12.4 ± 0.67 f
0.7	46.9±0.41^{e}	13.8 ±0.33^{e}
0.8	49.11± 0.10^{c}	10.4±0.21^{c}
IBA		
0.3	**49.8 ± 0.24^{d}**	**18.3 ± 0.32^{d}**
0.4	45.1 ± 0.66^{f}	16.8 ± 0.11^{f}
0.5	39.7 ± 0.71^{h}	14.1 ± 0.80 ^{h}
0.6	34.2 ± 0.71 ^{i}	12.4 ± 0.13 ^{i}
0.7	30.1 ± 0.56^{k}	11.5 ±0.56^{k}
0.8	29.2 ± 0.89 ^{l}	--

Data were collected after15 days of culture in MSB_5 medium supplemented with auxins; Best results are indicated in bold letters. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of roots per elongated shoot was recorded after four weeks of culture on MSB_5 medium supplemented with auxins.

Table 5: Organogenic callus induction from leaf explants of *Psoralea corylifolia* L.

Plant growth regulators (mg/L)	**Leaf explant**	
	Percentage of response	**Callus nature**
2,4 -D		
0.5	72.9 ±1.24 [c]	YGF
1.0	76.6 ± 1.36[b]	YGF
1.5	**84.8 ± 2.51 [a]**	**GC**
2.0	71.6 ± 2.45[e]	GF
2.5	63.6± 1.12 [f]	YGF
3.0	54.1±1.11 [i]	YF
KIN		
0.5	52.4 ± 0.51 [j]	YF
1.0	71.7 ± 0.23 [d]	YGF
1.5	62.5 ± 0.43 [g]	GF
2.0	55.5 ± 0.61 [h]	GF
2.5	48.4 ± 0.24 [k]	YG
3.0	36.5 ± 0.15 [l]	YG

GFC-Green Friable, GC-Green Compact, YGF-Yellowish Green Friable, YF-Yellowish Friable

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold.

Table 6: Multiple shoot formation from leaf derived organogenic callus of *Psoralea corylifolia* L.

Concentration of plant growth regulators (mg/L)	Leaf explant	
	Percentage of response	Mean number of shoots per callus
2,4-D		
0.5	53.6±0.15^{i}	5.2±0.15^{i}
1.0	61.5±0.50^{c}	7.3±0.11^{c}
1.5	**67.7±0.41^{b}**	**8.9±0.12^{b}**
2.0	58.4±0.21^{e}	6.5±0.22^{e}
2.5	51.5±0.39^{j}	5.1±0.11^{j}
TDZ		
0.5	49.6±0.58^{k}	4.9±0.30^{k}
1.0	**56.9±0.63^{f}**	**5.7±0.10^{f}**
1.5	55.1±0.27^{g}	5.5±0.12^{g}
2.0	43.2±0.55^{m}	4.3±0.21^{m}
2.5	35.7±0.35^{o}	2.8±0.12^{o}
KIN		
0.5	41.8±0.55^{n}	3.1±0.15^{n}
1.0	48.9±0.75^{l}	4.8±0.19^{l}
1.5	54.5±0.37^{h}	5.1±0.13^{h}
2.0	**68.7±0.56^{a}**	**9.9±0.35^{a}**
2.5	59.2±0.51^{d}	7.5±0.12^{d}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of shoot buds per explant and number of elongated shoots were recorded after four weeks of culture on MSB_5 medium supplemented with auxin and cytokinins.

Table 7: Effect of different concentrations of auxins on root induction of elongated shoots

Concentration of Plant growth regulators (mg/L)	Shoots	
	Percentage of response	Mean number of roots per shoot
IAA		
0.2	44.5±0.13^{p}	3.8 ±0.14^{p}
0.4	61.6±0.22^{g}	7.5±0.19^{g}
0.6	**67.1±0.67^{d}**	**9.0±0.12^{d}**
0.8	59.2±0.23^{i}	6.1±0.41^{i}
2.0	48.6±0.56^{q}	4.1±0.17^{q}
1.2	38.3±0.12^{r}	2.1±0.19^{r}
IBA		
0.2	51.6±0.23^{m}	4.9±0.11^{m}
0.4	53.9±0.11^{l}	5.9±0.19^{l}
0.6	63.5±0.30^{f}	7.5±0.11^{f}
0.8	**73.8±0.14^{a}**	**12.9±0.19^{a}**
2.0	69.8±0.49^{c}	10.1±0.28^{c}
1.2	49.9±0.50^{n}	4.8±0.19^{n}
NAA		
0.2	59.6±0.18^{h}	7.0±0.13^{h}
0.4	65.9±0.14^{e}	8.9±0.28^{e}
0.6	**69.8±0.94^{b}**	**10.1±0.11^{b}**
0.8	58.4±0.76^{j}	5.6±0.17^{j}
2.0	56.8±0.51^{k}	5.1±0.10^{k}
1.2	48.6±0.70^{o}	2.5±0.19^{o}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of roots per elongated shoot was recorded after four weeks of culture on MSB_5 medium supplemented with auxins.

Table 8: Organogenic callus induction from cotyledon explant of *Psoralea corylifolia* L.

Plant growth regulators (mg/L)	Cotyledon	
	Percentage of response	Nature of callus
BAP		
0.5	23.87 ± 0.11^{ij}	GF
1.0	37.12 ± 0.22^{d}	GF
1.5	**28.9 ± 0.10^{f}**	**GC**
2.0	15.0 ± 0.19^{l}	GC
2.5	18.5 ± 0.11^{k}	BYC
IBA		
0.5	24.4 ± 0.24^{i}	GC
1.0	28.6 ± 0.17^{h}	GF
1.5	**42.9 ± 0.62^{b}**	**GF**
2.0	13.2 ± 0.50^{m}	GC
2.5	9 ± 0.210^{n}	GC
2,4-D		
0.5	37.8 ± 0.16^{de}	GF
1.0	**53.2 ± 0.29^{a}**	**GC**
1.5	39.4 ± 0.18^{c}	GF
2.0	28.8 ± 0.10^{g}	GC
2.5	19.3 ± 0.114^{k}	GC

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p<0.05$). Best results are indicated in bold.

Table 9: Multiple shoot formation from cotyledon derived callus of *P. corylifolia* L.

Concentration of plant growth regulators (mg/L)	Percentage of response	Mean number of shoots per callus
2iP		
0.5	89.0±0.72^{c}	9.2±0.12^{c}
1.0	78.5±0.65^{i}	6.4±0.15^{i}
1.5	**98.9±0.88^{a}**	**17.6±0.19^{a}**
2.0	92.7±0.96^{b}	12.8±0.33^{b}
2.5	88.4±0.72^{d}	8.9±0.16^{d}
BAP		
0.5	79.4±0.55^{g}	6.9±0.17^{g}
1.0	78.9±0.55^{h}	6.3±0.45^{h}
1.5	75.5±0.40^{j}	5.8±0.15^{j}
2.0	**85.7±0.58^{e}**	**7.6±0.63^{e}**
2.5	82.3±0.91^{f}	7.1±0.10^{f}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of shoot buds per explant and number of elongated shoots were recorded after four weeks of culture on MSB_5 medium supplemented with cytokinins.

Table 10: Effect of different concentrations of auxins on root induction of elongated shoots

Concentration of plant growth regulators (mg/L)	Percentage of response	Mean number of roots/ shoot
IAA		
0.2	82.8±0.15^{o}	8.6±0.15^{o}
0.4	89.1±0.16^{j}	12.3±0.17^{j}
0.6	90.6±0.19^{g}	13.1±0.18^{g}
0.8	**93.5±0.10^{c}**	**15.6±0.12^{c}**
1.0	90.2±0.32^{h}	13.1±0.14^{h}
IBA		
0.2	**93.9±0.24^{b}**	**16.1±0.18^{f}**
0.4	91.3±0.15^{f}	15.8±0.53^{b}
0.6	87.1±0.18^{l}	13.9±0.30^{l}
0.8	89.5±0.10^{i}	12.2±0.11^{i}
1.0	85.2±0.11^{n}	9.5±0.10^{n}
NAA		
0.2	85.9±0.16^{m}	9.6±0.13^{m}
0.4	**94.3±0.35^{a}**	**17.9±0.18^{a}**
0.6	91.9±0.50^{d}	16.2±0.15^{d}
0.8	88.5±0.10^{k}	12.1±0.10^{k}
1.0	91.7±0.15^{e}	14.2±0.17^{e}

Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05). Best results are indicated in bold. Average number of roots per elongated shoot was recorded after four weeks of culture on MSB_5 medium supplemented with auxins.

3.2 Elicitor treatment

The present study was carried out to test MeJ, SA, JA and $AgNO_3$ for enhancement production of psoralen in adventitious root culture of *P. corylifolia*. The leaf explants were inoculated on mMS medium supplemented with auxins at different concentrations. The medium containing 3% sucrose with NAA (0.5 mg/L), IAA (1.0 mg/L) and IBA (1.5 mg/L) were found as the optimum concentration for high number of root induction. This result suggested the synergistic effect of auxins on efficient root induction which is previously reported by Baskaran and Jayabalan (2009) for establishment of an efficient adventitious root culture system from hypocotyl explants of *P. corylifolia* on MS salts and B_5 vitamins medium containing 3% sucrose, 4 µM IAA and NAA.

At the 28^{th} day liquid culture, roots treated with elicitors exhibited visible changes in root morphology such as color and texture of the root and increased weight. Table 4.1 indicates SA at 150 µM/L showed bulging of roots with growth index of 1.01 and 2.01 fold higher in root weight and MeJ at 30 µM/L exerted growth index of 1.76 and fold increase of 2.76 (Fig 7, Table 11&12). Table 4.2 indicates that the best result was observed in 200 µM/L of JA treated roots with a growth index of 1.84 and 2.84 fold increase in root weight. In contrary to this, $AgNO_3$ at 150 µM/L gave 1.47 fold increase and 0.47 growth index (Fig 12, Table 13&14). Beyond the optimum concentrations mentioned above, all elicitors resulted decrease in root growth index and fold increase.

3.2.1 HPLC analysis of Elicitor treated root sample

Psoralen standard chromatogram showed peak at Retention Time 20.628 min

HPLC analysis of methanolic extract of MeJ treated root samples showed a single peak at the retention time (RT) 21.265 min (Fig 10) and estimated concentration of psoralen was 3.73 mg/mL. SA treated root sample showed peak at RT 21.651 min (Fig 11) and estimated concentration of psoralen was 0.15 mg/mL. Methanolic extract of JA treated root samples showed peak at the RT 21.501 min (Fig 13) and estimated concentration of psoralen was 8.99 mg/mL. $AgNO_3$ treated root sample showed peak at RT 21.181 min (Fig 14) and estimated concentration of psoralen was 1.047 mg/mL. Methanolic extract of root sample from naturally grown plant gave peak at RT 21.312 min (Fig 9) and estimated concentration of psoralen was 0.56 mg/mL. Estimation of psoralen content in root samples was done as follows

Estimation of psoralen from naturally grown plant

Sample concentration = $\frac{15098}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$

0.0563×0.0002×50 = 0.00015×1000 = 0.56 mg/mL

Estimation of psoralen from MeJ treated roots

Sample concentration = $\frac{99747}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$

0.37376×0.0002×50=0.00373×1000 = 3.73 mg/mL

Estimation of psoralen from SA treated roots

Sample concentration = $\frac{4018}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$

0.01505×0.0002×50 = 0.00015×1000 = 0.15 mg/mL

Estimation of psoralen from JA treated roots

Sample concentration = $\frac{240083}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$

0.89962×0.0002×50 = 0.008996 g ×1000 = 8.99 mg/mL

Estimation of psoralen from $AgNO_3$ treated roots

Sample concentration = $\frac{27957}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$

0.104759×0.0002×50 = 0.001047 g ×1000 = 1.047 mg/mL

From the above observations, elicitor treated *in vitro* root samples exhibited higher concentration of psoralen when compared to root samples obtained from naturally grown plant. The present result is in accordance with the previous report of Baskaran and Jayabalan (2008) in which HPLC analysis of psoralen was performed from both *in vitro* and *ex vitro* grown plants of *P. corylifolia* plants resulted that psoralen content was higher from *in vitro* grown plants when compared to naturally grown plants.

In this study, four elicitors namely MeJ, SA, JA and $AgNO_3$ were used to increase psoralen content in adventitious root culture of *P. corylifolia*. In which, JA treated roots showed higher accumulation of psoralen content (8.99 mg/mL) followed by MeJ treated root samples (3.73 mg/mL) when compared to other elicitors tested. Addition of MeJ and SA increased root growth which subsequently accumulated the psoralen content in adventitious root culture of *P. corylifolia*. Amit Shinde *et al.*, (2009, 2010) reported that addition of SA at 1 mM concentration stimulated high accumulation of isoflavones in hairy root culture of

P. corylifolia after 2 days of elicitation, but further increasing the concentration of SA beyond the optimal concentration and incubation period, reduced root growth and psoralen accumulation were observed. Elicitor treatment has been proved to enhance the production of secondary metabolites in adventitious root culture of some other medicinal plants also (Sivanandhan and Ganapathi, 2011). MeJ and SA were used as elicitors for higher production of withanolides in adventitious root culture of *Withania somnifera* (Ganeshan Sivanandhan and Ganapathi, 2012).

Addition of JA and $AgNO_3$ enhanced root growth index which subsequently increased the psoralen content in adventitious root culture of *P. corylifolia*. Bulging of root was observed after 8 h of contact time on JA at 200 µM/L and $AgNO_3$ at 150 µM/L concentrations showed profuse root growth at 8 h of contact time. JA has been previously applied as elicitors in root cultures of *Brugmansia candida* in which JA and $AlCl_3$ showed a positive effect on release of two alkaloids scopolamine and hyoscyamine. The most important effect was induced by JA at the highest concentration and after 24 h of exposure which promoted the release of hyoscyamine (~1200%) (Spollansky *et al.,* 2000). The present result is in accordance with the earlier observation made by Anja *et al.,* 2010 in which JA at 200µM was found to be optimum for increased production of Glucosinolate in hairy root cultures of *Sinapis alba* with 2 weeks of cultivation. From this study it was observed that JA increased the growth index of adventitious roots better than $AgNO_3$. Visible changes in root morphology such as colour and texture of the root and increased in fresh weight were noticed after elicitation period.

3.3 *Agrobacterium rhizogenes*-mediated transformation

3.3.1 Development of hairy roots

The wild type strain (A4) of *A. rhizogenes* was used to induce hairy roots from leaf explant. In general, *Agrobacterium* strains which induced hairy roots at the site of infection were evaluated as virulent strains. The infection process is a complex series of events in which chemotaxis is the first step of the infection process and attachment of *Agrobacterium* to the plant cell surface (Binns and Thomashow, 1988). The next step is bacterial colonization and attachment to plant cells at the infection site, which leads to the insertion of T-DNA borders of the root inducing plasmid of the bacterium into the plant genome (Binns and Thomashow, 1988). Hairy roots were induced from leaf explants at the site of infection after 4 weeks. Whereas, hairy root induction was not observed in control but explants

remained green. The transformation frequency is influenced mainly by two factors such as bacterial inoculums and the co-culture time (Karmarkar and Keshavachandran 2001).

In present study, roots induced by strain A4 were thin with profuse lateral roots, fast growing, whitish in colour with numerous hairs on the surface. Growth of hairy roots was high with 36 h co-culture period, and yielded maximum number of roots (12.6 per explant) after 4 weeks of culture (Table 15).

Table 11: Effect of Methyl Jasmonate on adventitious root growth of *P. corylifolia*

Concentration of Methyl Jasmonate (µM/L)	Initial fresh Weight (g)	Final fresh Weight (g)*	Growth Index	Fold Increase
0	3	3.78 ± 0.15^{e}	0.26	1.26
10	3	5.03 ± 0.15^{d}	0.67	1.67
20	3	5.96 ± 0.15^{b}	0.98	1.98
30	**3**	**8.30 ± 0.2^{a}**	**1.76**	**2.76**
40	3	5.50 ± 0.2^{c}	0.83	1.83

*values are represented as Mean ± SD

Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by DMRT. Best results are indicated in bold.

Table 12: Effect of Salicylic Acid on adventitious root growth of *P. corylifolia*

Concentration of Salicylic Acid (µM/L)	Initial fresh Weight (g)	Final fresh Weight (g)*	Growth Index	Fold Increase
0	3	3.78 ± 0.15^{e}	0.26	1.26
50	3	3.99 ± 0.15^{d}	0.33	1.33
100	3	4.3 ± 0.20^{c}	0.43	1.43
150	**3**	**6.03 ± 0.15^{a}**	**1.01**	**2.01**
200	3	5.5 ± 0.30^{b}	0.83	1.83

*values are represented as Mean ± SD

Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by DMRT. Best results are indicated in bold.

Table 13 : Effect of Jasmonic Acid on adventitious root growth of *P. corylifolia*

Concentration of Jasmonic Acid (µM/L)	Initial fresh Weight (g)	Final fresh Weight (g) *	Growth Index	Fold increase
0	3	3.78 ± 0.15^{d}	0.26	1.26
50	3	5.16 ± 0.35^{d}	0.72	1.72
100	3	5.16 ± 0.35^{d}	0.72	1.72
150	3	6.08 ± 0.36^{c}	1.02	2.02
200	**3**	**8.54 ± 0.36^{a}**	**1.84**	**2.84**
250	3	7.40 ± 0.41^{b}	1.46	2.46

*values are represented as Mean ± SD

Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by DMRT. Best results are indicated in bold.

Table 14: Effect of Silver nitrate on adventitious root growth of *P. corylifolia*

Concentration of $AgNO_3$ (µM/L)	Initial fresh Weight (g)	Final fresh Weight (g) *	Growth Index	Fold increase
0	3	3.78±0.15	0.26	1.26
50	3	3.84±0.11	0.28	1.28
100	3	3.98±0.58	0.32	1.32
150	**3**	**4.42±0.11**	**0.47**	**1.47**
200	3	4.08±0.58	0.36	1.36
250	3	3.82±0.16	0.27	1.27

*values are represented as Mean ± SD

Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by DMRT. Best results are indicated in bold.

3.3.2 Bioreactor culture

3.3.2.1 Effect of inoculums density and aeration volume on biomass and psoralen production

Bioreactor cultures are used to enhance biomass and bioactive compound production in plant cell or organ culture (Jeong *et al.*, 2009a,b). To determine the optimum biomass production and to evaluate the accumulation of psoralen content, hairy roots [15g/L FW (0.8 g/L DW)] were inoculated onto bioreactor. The cultures were incubated in the dark at 25 °C and then amount of biomass accumulated psoralen content was then estimated after 5 weeks of culture (Fig 15). Previous studies have indicated that while the optimal inoculum densities for cell cultures exceed 60 g/L. Compared to adventitious root cultures (10 g/L) because of the slow growth (Jeong *et al.*, 2009a,b; Lee *et al.*, 2006; Thanh *et al.*, 2006). Similarly, an inoculum density of 15 g/L was suitable for generating the optimum hairy root biomass in the case of *P. corylifolia*.

Optimizing aeration volume is one conventional way of controlling the gaseous composition, which can affect root growth. The growth index of the root biomass increased slightly with different aeration volumes (2-10 LPM). Supply of aeration at a high volume into a bioreactor may cause excessive shear stress to roots and thicker cell walls (Fischer and Alfermann, 1995). Accumulation of root biomass was higher at aeration volume of 8 LPM than at the other aeration volumes. Increasing the aeration volume improves oxygen transfer and the mixing efficiency of explants with the culture medium. In the present study, a poor aeration volume (2 LPM) resulted in stunted root growth and stimulated root senescence because of low oxygen transfer and mixing ratio. Similarly, the highest aeration volume (10 LPM) inhibited accumulation of biomass, probably because of physiological damage due to the excessive agitation and shear stress (Meijer *et al.*, 1993). In general, cultured cells require less oxygen than microorganisms because of their larger vacuoles and metabolism (Min *et al.*, 2007). Each plant species requires a different aeration volume and responds differently to shear stress. In the present study, the least root death and highest accumulation of root biomass were achieved at an aeration volume of 8 LPM (Table 16). This suggests that 8 LPM is optimal for higher accumulation of *P. corylifolia* hairy roots, because it supplies sufficient O_2 for anabolism, prevents accumulation of large amounts of CO_2 inside the bioreactor, and does not cause shear stress or root physiological disorders such as changing to a dark color or inhibiting root elongation. Studies in which the adventitious roots of *Scopolia parviflora* (Min *et al.*, 2007) and *E. purpurea* (Jeong *et al.*, 2009a,b) were cultured have reported that

root growth and metabolism in these plants were affected by shear stress. It is possible that various chemical factors (such as salt strength, nitrogen sources, and sucrose concentration) had a greater influence on both root growth and accumulation of bioactive compounds than the physical factors such as initial inoculum density and aeration volume.

From the above results of the present study, 8 LPM was found to be optimal aeration volume to prevent the stripping of essential gaseous components and to supply adequate air to maximize the accumulation of root growth and psoralen in the hairy roots of *P. corylifolia*.

3.3.2.2 Estimation of psoralen

The psoralen content was analyzed in hairy roots which exhibited best growth index (Table 5.2). Psoralen content in hairy roots was estimated to be 3.54 mg/mL at RT 21.165 (Fig 16) by comparing peak area and peak height of the crude sample with that of authentic psoralen (Fig 5.1).

$$\text{Sample concentration} = \frac{99747}{266869} \times \frac{0.002}{10} \times \frac{5}{0.1}$$

The methanolic extract was subjected to preparative HPLC and the psoralen peak was collected at RT 20.172 through a fraction collector for NMR analysis. Suspension-grown roots often produce higher concentrations of bioactive compounds (Panichayupakaranant and Tewtrakul 2002). From a biotechnological point of view, the productivity of psoralen has practical value due to spare the over-exploitation of the naturally grown plants. Hence the present work has provided a system of hairy root cultures of *P. corylifolia*, which can be recommended to approach for production of psoralen in a large scale level.

3.3.2.3 FTIR analysis

FTIR analysis was revealed the presence of different functional groups ranging from C-H stretching (2858 cm-1), N-O asymmetric stretching (1512 cm-1) which are responsible for the medicinal properties of the plant (Table 17, Fig 17).

3.3.2.4 NMR

The ^{1}H NMR of HPLC purified compound yielded a molecular formula C_{11} H_6 O_3 and the compound was identified as Psoralen. In the ^{1}H NMR spectra, almost all of the signals are well resolved and are distributed in the region from 7.057 to 7.135 (Table 18, Fig 18).

Table 15: *A. rhizogenes* (A4) mediated hairy root formation in leaf explant

Co-cultivation period (h)	Frequency of transformation	Number of hairy roots/explant*
12	36	1.9±0.05[d]
24	42	2.8±0.06[c]
36	**68**	**12.6±0.15[a]**
48	28	5.4±0.26[b]

Values are mean of 20 explants per treatment and repeated thrice. Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by Duncan's multiple range test (DMRT).

Table 16: Effect of different aeration volumes on hairy root biomass

Aeration (LPM)	Final fresh weight (g)	Growth Index	Fold increase
2	58±0.62[e]	4.8	3.8
4	72±0.45[d]	3.8	4.8
6	95±0.88[c]	5.3	6.3
8	**145±0.53[a]**	**8.6**	**9.6**
10	110±0.73[b]	6.3	7.3

Mean values within a column followed by different letters are significantly different from each other at 5% level comparison by DMRT. Best results are indicated in bold. Hairy root biomass at 15g/FW was cultured in bioreactor at different aeration volumes.

Table 17: FTIR analysis of *Psoralea corylifolia* L. leaf extract

Frequency range	Intensity	Type and group	Bond
3323	m	1^o,2^o amines,amides	N-H Str
2858	s	Aromatics	=C-H Str
2924	m	Alkanes	C-H Str
1608	s	Carboxylic acid	C=O Str
1512	s	Nitro compounds	N-O assymentric Str
1020	s	Sulphites	C=H bend
1170	a	Aliphatic amines	C-N str
970	a	Aliphatic amines	C-N str

Table 18: NMR analysis

C Numbering	Chemical shift	
	^{1}H δppm	^{13}C δppm
H1	6.79 - 6.85	169.77
H2	6.98 - 7.09	157.86
H3	7.11 - 7.14	144.86
H4	7.16 - 7.19	130.04
H5	7.21 -7.30	128.84
H6	7.21 - 7.30	123.75
H7	--	122.24
H8	--	116.09
H9	--	113.29
H10	--	108.75

Annexure- II

SUMMARY OF THE FINDINGS:

Psoralea corylifolia Linn.is an endangered medicinal plant used in Folk, Siddha and Ayurvedic system of medicine. It is distributed in the tropical region of the world. Number of bioactive compounds have been reported earlier from fruits, seeds and roots of *P. corylifolia*. The major compounds of this plant are psoralen, angelicin, psoralone, isopsoralone, bavachin and daidzein. Psoralen is a pharmaceutical important compound which is used for the photochemotherapy of vitiligo and skin diseases such as psoriasis, mycosis fungoides and eczema. The plant is also used as laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic in febrile conditions. It is specially recommended for the treatment of leucoderma, leprosy and inflammatory diseases of the skin in the form of a paste or ointment. Also, it exhibits antitumour, antibacterial, antifungal and antioxidative activities. Many Indian pharmaceutical industries have used seeds of *P. corylifolia* as a raw material to produce medicines and Ayurvedic skin care soaps. Accordingly, *P. corylifolia* was selected for this study and succeeded with the following results.

Seed germination

- A simple and effective protocol was developed in the present study to overcome the seed dormancy and to improve the frequency of seed germination.
- The seed germination rate was increased by using hot water and acid treatment.
- The treated (hot water and acid) seeds were sterilized and then inoculated into sterile moistened cotton. The cultures were maintained in dark condition for 48 h at 25±2°C followed by 16 h light and 8 h dark photoperiod condition.
- The highest percentage of germination (70%) and survival rate were achieved in 70°C for 15 min compared to control and other treatments.
- The underlying mechanism of hot water treatment method involved in breaking seed dormancy and improved seed germination.
- The hot water treatment method could be adopted as a good alternative technique for obtaining higher percentage of seed germination.

***In vitro* regeneration**

- *In vitro* regeneration of *P. corylifolia* was achieved through direct and indirect organogenesis.

Direct organogenesis

- mMS medium supplemented with 2.0 mg/L BAP gave best result for multiple shoot formation from shoot tip explant with a mean of 16.3± 0.24 shoots per explant with 82.2 % of response after four weeks of culture.
- mMS medium supplemented with 0.8 mg/L IAA was the most effective for root induction with mean of 18.6 ± 0.34 roots per explant with 82.6 % of response.
- Nodal explants resulted multiple shoot production with a combination of KIN and BAP at 0.3 and 0.4 mg/L with 44.5% of response and mean number of 19.1±0.11 shoots per explant. NAA at 0.5 mg/ml was found to be suitable for highest root induction with 72.2 % of response with mean number of 15.2 roots per shoot.

Indirect organogenesis

- Higher percentage (84.8%) of response of green compact callus was obtained from leaf explants on mMS medium supplemented with 1.5 mg/L 2,4-D than other explant and treatments.
- Multiple shoots were obtained on mMSmedium supplemented with Kin (2 mg/L) for proliferation and elongation. Rooting of the elongated shoots was best obtained with 0.4 mg/l NAA with 94.3% of root induction and mean number of 17.9 roots/shoot.

Adventitious root culture

- Production of adventitious roots was obtained from leaf explants on mMS medium containing 3% sucrose, 0.8% agar and supplemented with different concentrations of NAA, IAA and IBA.
- Combination of NAA (0.5 mg/L)+IAA (1.0 mg/L)+IBA (1.5 mg/L) was most effective concentration for higher root biomass production.
- Abiotic elicitors (MeJ, JA, SA and $AgNO_3$) along with optimum concentration of auxins (0.5mg/L NAA, 1.0mg/L IAA and 1.5mg/L IBA) increasedpsoralencontent at 28^{th} days of adventitious root culture.
- Higher production of psoralen content (8.99 mg/mL) was obtained in 200 µM/L of JA treated roots compared to control and other treatments of elicitors.

Hairy root induction

- Hairy roots were achieved with *A. rhizogenes* (A4 strain) leaf explants after 4 weeks of culture. The highest transformation frequency (68%) was achieved with co-cultivation of 36-h.
- Highest root biomass (95 g dry weight) was obtained from bubble type bioreactor with inoculum density of 15 g/L FW and 8 LPM.
- Higher content of psoralen (3.54 mg/mL) was obtained in hairy roots with RT of 21.165 min.
- The psoralen content was confirmed by ^{1}H NMR with molecular formula of $C_{11}H_6O_3$.
- In the present investigations, high efficiency of seed germination and *in vitro* plant regeneration was achieved for conservation of *P. corylifolia*. The pharmaceutical important compound psoralen was enhanced and analysed by using different techniques (HPLC,FTIR and NMR) isolated, characterized and *in vitro* root cultures (adventitious and hairy root cultures).

CONCLUSION

- The present research work offers higher frequency of seed germination by hot water treatment and efficient *in vitro* plant regeneration system from shoot tip explant of *P. corylifolia*.
- The pharmaceutical important compound, psoralen was enhanced through adventitious and hairy root cultures and characterized by HPLC,FT-IR and NMR. The developed protocol can be further extended for commercial production of psoralen for pharmaceutical applications

Annexure – III

Contribution to the society

Medicinal plants are natural sources of alkaloids and chemical substances which are being used to cure a variety of diseases in man. *Psoralea corylifolia* L. is an important plant used in Folk, Siddha and Ayurvedic system of medicine. It is an endangered and rare herbaceous medicinal plant distributed in the tropical region of the world. Many Indian pharmaceutical industries have used *P. corylifolia* as a raw material to produce medicines and Ayurvedic skin care soap (Baskaran and Jayabalan, 2007). Among several bioactive compounds of this plant, psoralen occupies a prominent position due to its several biomedical applications. It is specially recommended for the treatment of leucoderma, leprosy, psoriasis and inflammatory diseases of the skin and prescribed both for oral administration and external application in the form of a paste or ointment (Anonymous,1988 ; Orient longman,1996). Development of alternative tools for the production of useful compounds, such as plant tissue cultures, could decrease the danger of extinction of this valuable plant.

Tissue culture biotechnology can aid to provide uniform plant source. The development of a fast growing root system would offer unique opportunities for producing drugs without having to depend on field cultivation which is prone to show variability (Sudha and Seeni, 2001). Available reports clearly demonstrated the existence of variability in morphology as well as chemical constituents and so commercial production would be much more practical with bioreactor cultures, which has not yet been convincingly demonstrated in this most important medicinal plant.

Through this work we have standardized and established protocols for mass production of psoralen through adventitious and hairy root culture systems along with elicitor treatments. These techniques will certainly help to protect this valuable medicinal plant from over exploitation. More importantly, these protocols can be adopted for large scale production of psoralen through bioreactor culture to meet the demand of pharmaceutical industries which are keen to develop plant based medicinal drugs. This will create a possibility of cost effective production of psoralen for treating several diseases. The developed protocols can be further applied to produce other valuable medicinal compounds of *P. corylifolia* which will expand the market of novel plant based drugs.

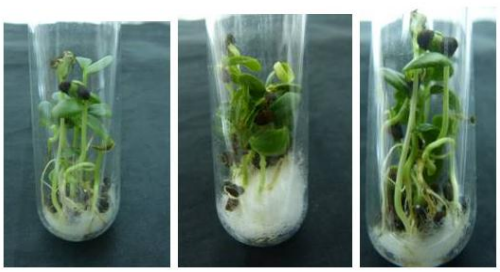
Fig.1 *Psoralea corylifolia* L. grow field condition in Bharathidasan University

Fig.2 Seed germination of *Psoralea corylifolia* L.

***P. corylifolia* seeds**

***P. corylifolia* plant**

***In vitro* seedlings**



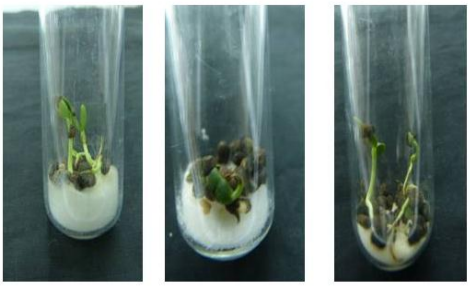
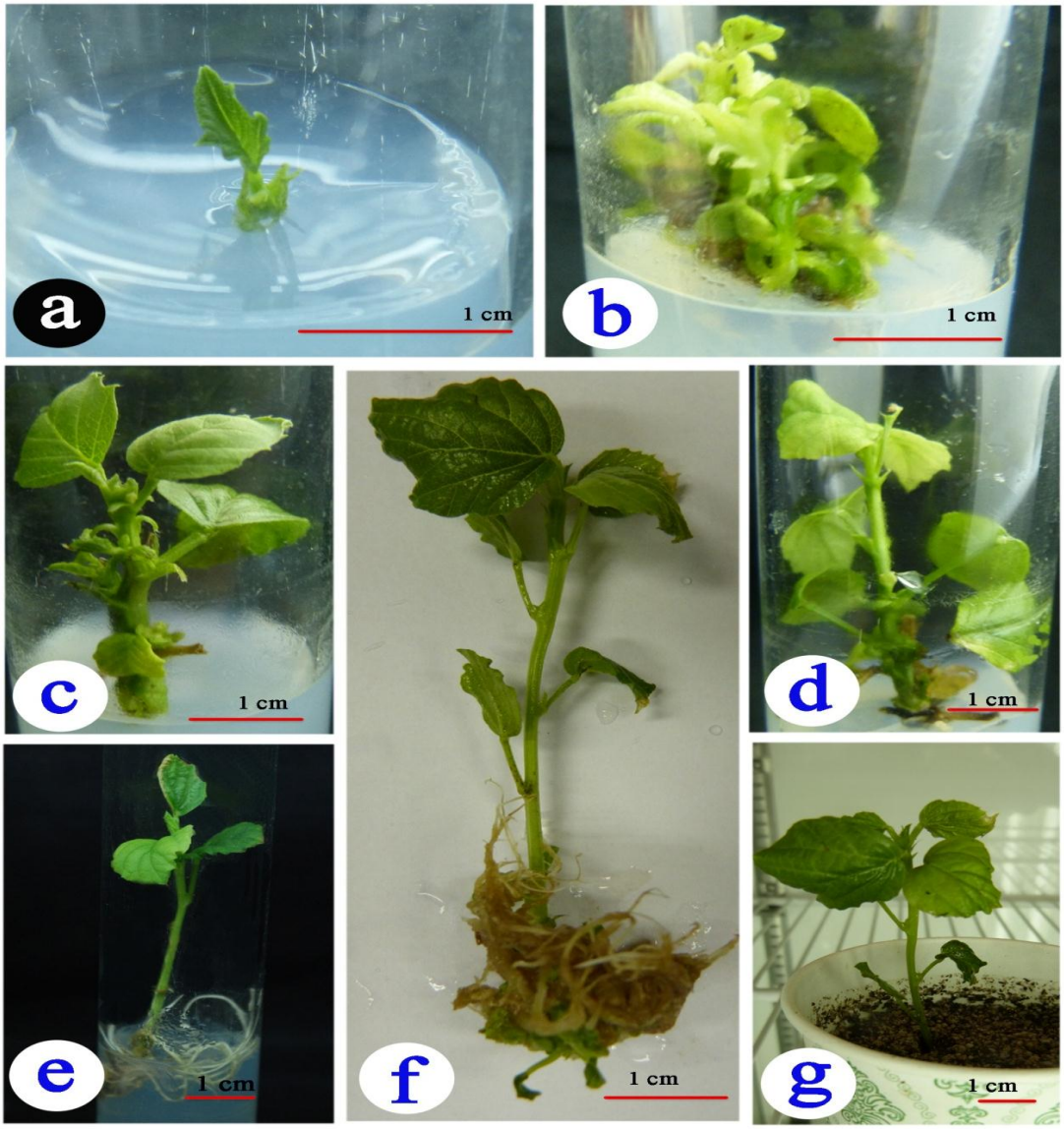
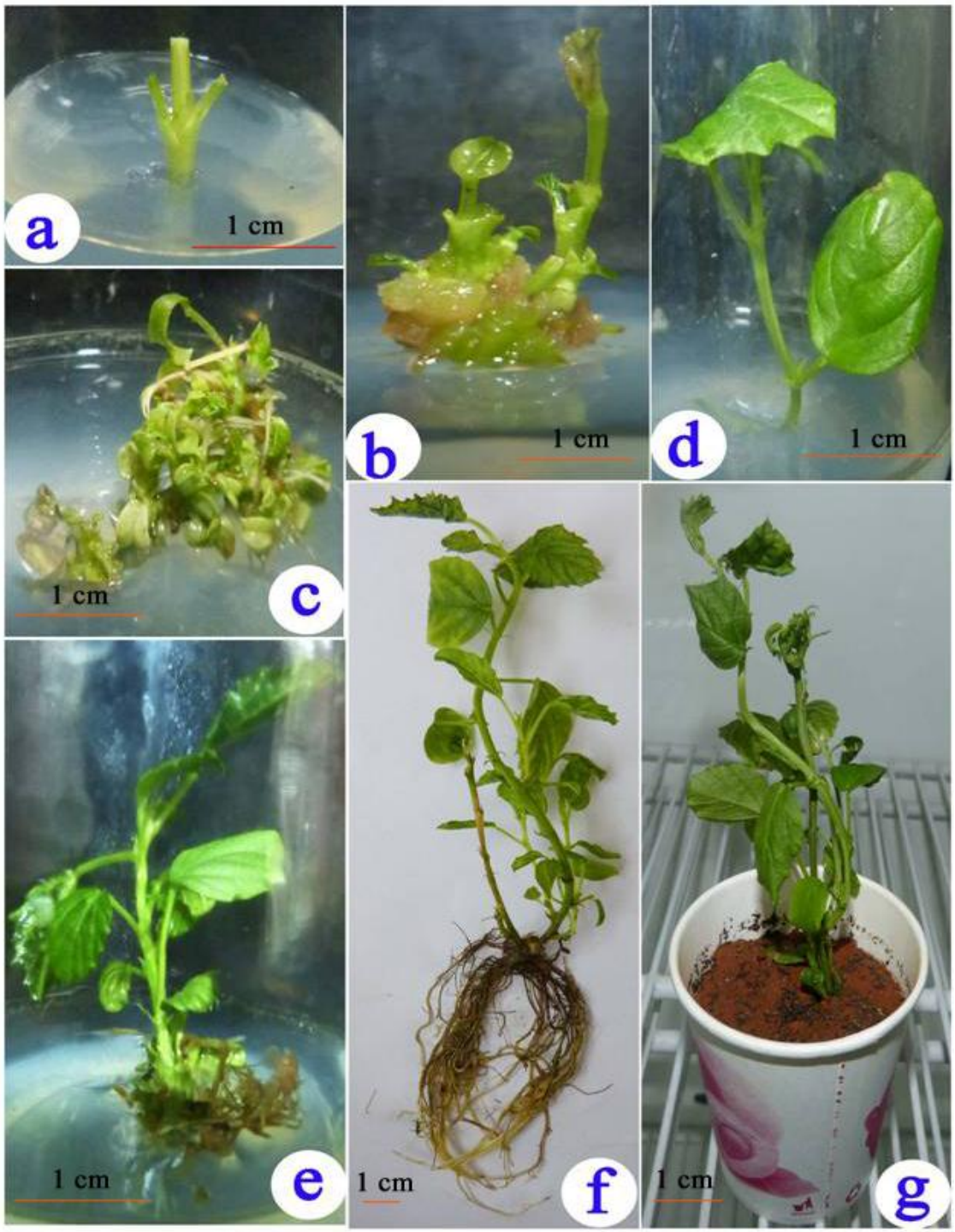


Fig. 3 Multiple shoot induction and proliferation of *Psoralea corylifolia* L. from shoot tip explant on MSB_5 medium



a.Shoot tip cultured on MSB_5 medium containing plant growth regulators, 6% sucrose and Jasmonic Acid, b. Multiple shoot formation and proliferation, c & d. Shoot elongation, e. Rooting of elongated shoot. f. Well developed rooted plant, g. Hardening in plant growth chamber.

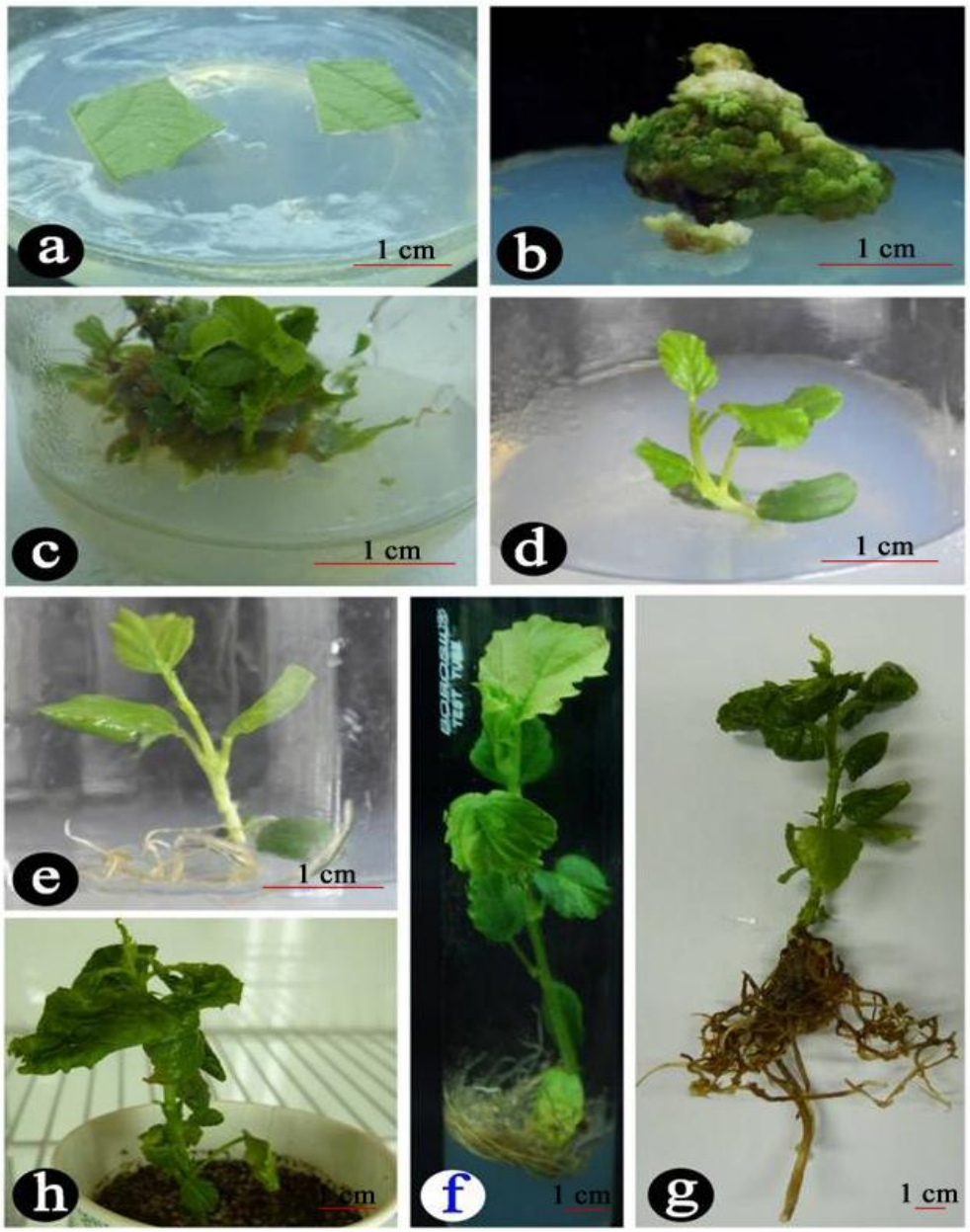
Fig. 4 Direct organogenesis from nodal explant of *Psoralea corylifolia* L.



a. Nodal explant, b. Multiple shoot initiation, c. Proliferation of multiple shoots, d. Shoot elongation, e. Root initiation, f. Well developed roots, g. Hardened plantlet

Figure 5

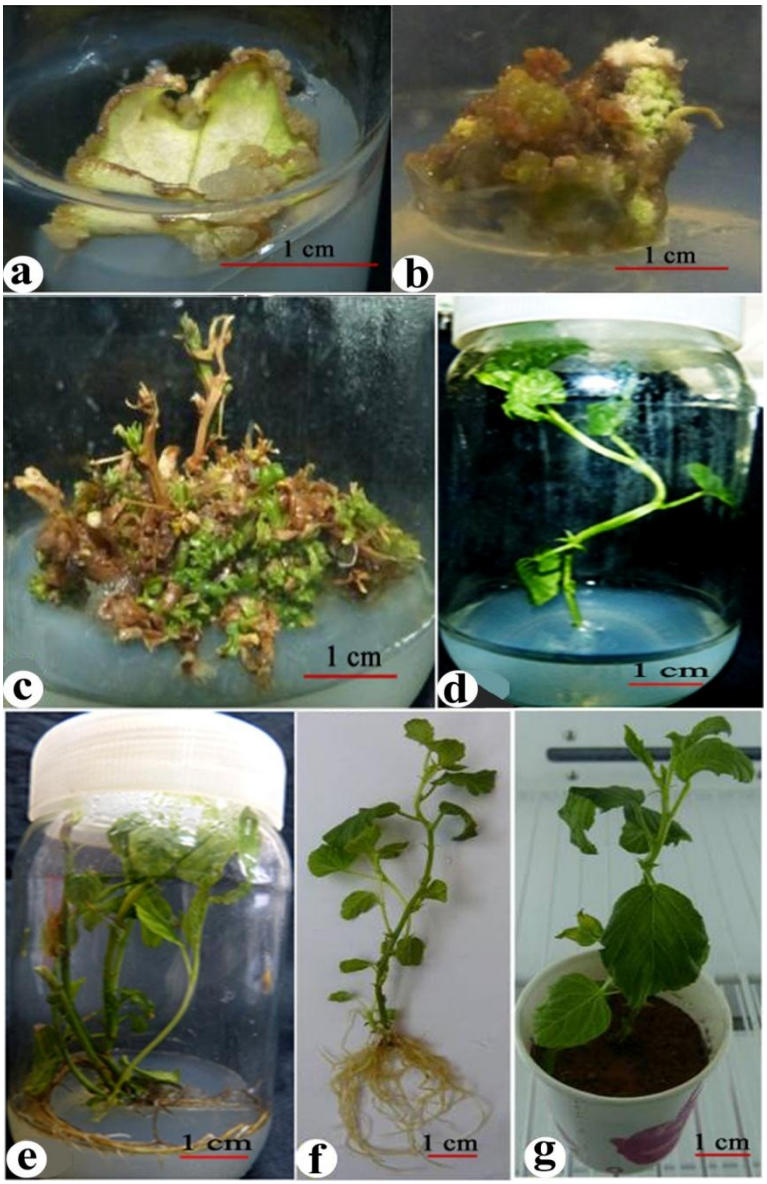
Indirect organogenesis from leaf explant of *Psoralea corylifolia L.*



a. Leaf explant, b. Callus proliferation, c. Multiple shoot induction, d. shoot Elongation, e. Root initiation, f &g. Well developed roots, h. Hardened Plantlet

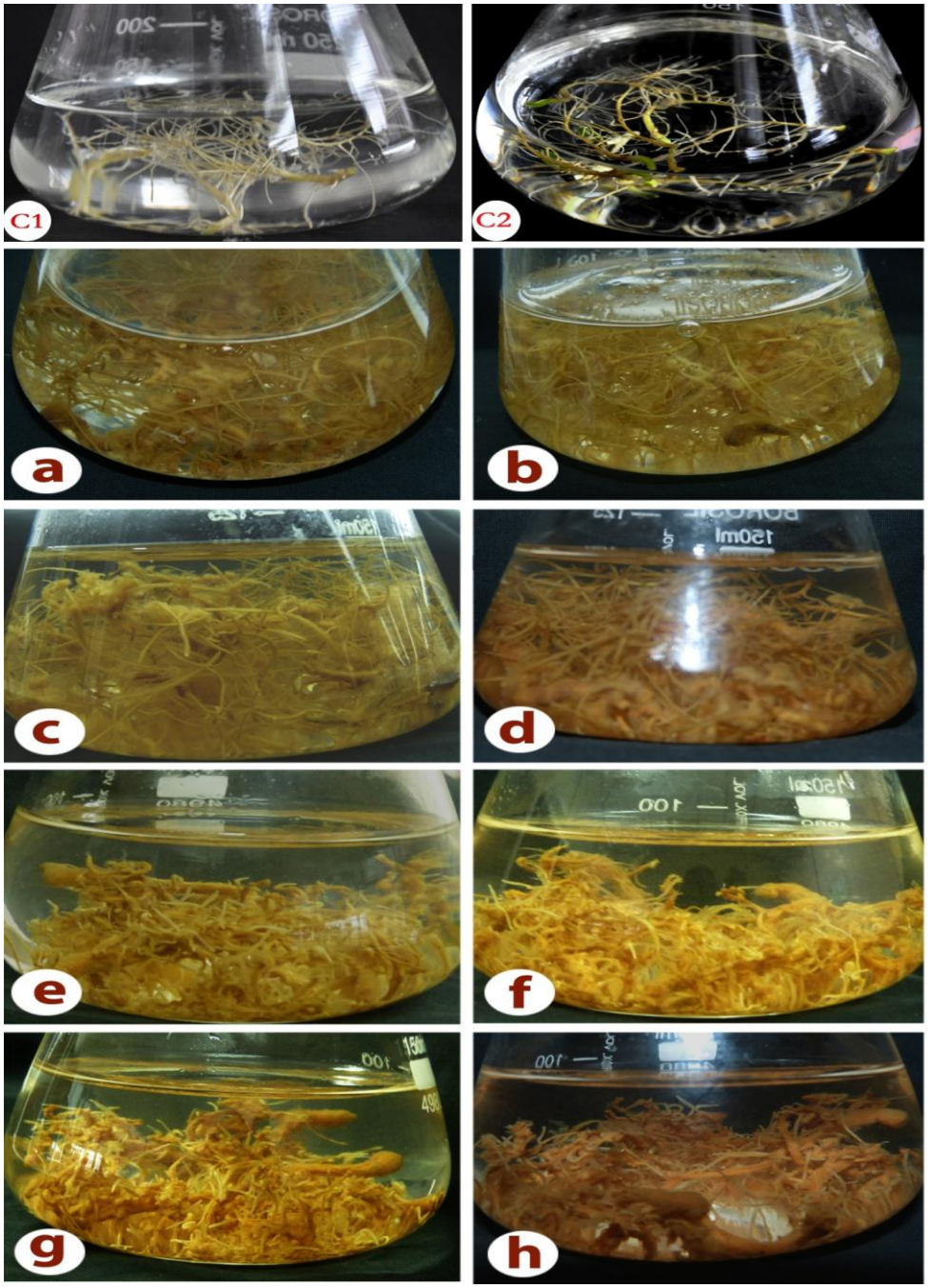
Figure 6

Indirect organogenesis from cotyledon explant of *Psoralea corylifolia* L.



a. Callus induction from cotyledon explant. b. Well developed organogenic callus, c. Multiple shoot production, d. Shoot elongation, e, Rooting of elongated shoot, f. Well rooted plantlet, g. Hardened Plantlet

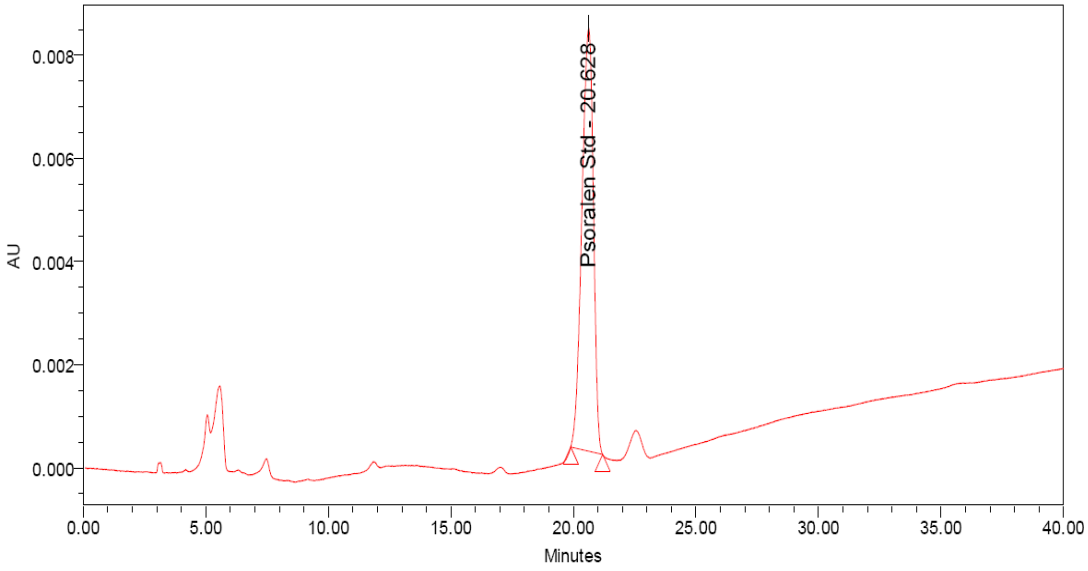
Fig: 7 Effect of different concentration of elicitor treatment (28th day old culture and 8 h contact time) **on *in vitro* adventitious root growth of *Psoralea corylifolia* L.**



a to d - MeJ treated adventitious root (a, 10 µM/L; b, 20 µM/L; c, 30 µM/L; d, 40 µM/L, C1 Control)

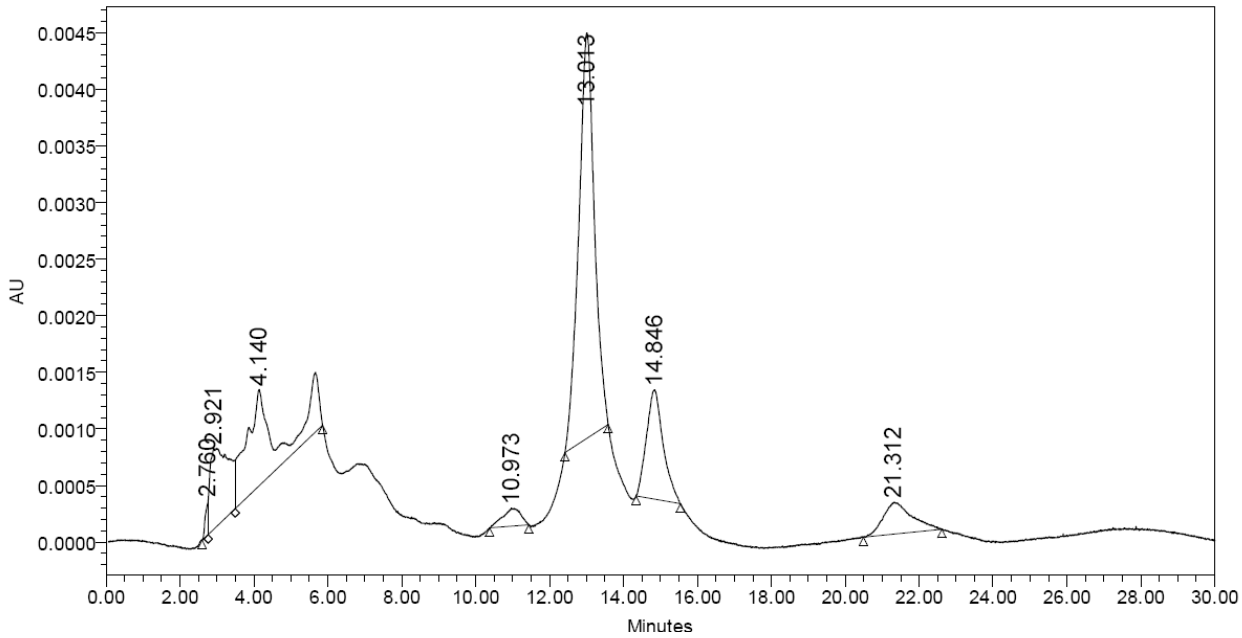
e to h - SA treated adventitious root (e, 50 µM/L; f, 100 µM/L; g, 150 µM/L; h, 200 µM/L, C2 control)

Fig: 8 Psoralen Standard Chromatogram



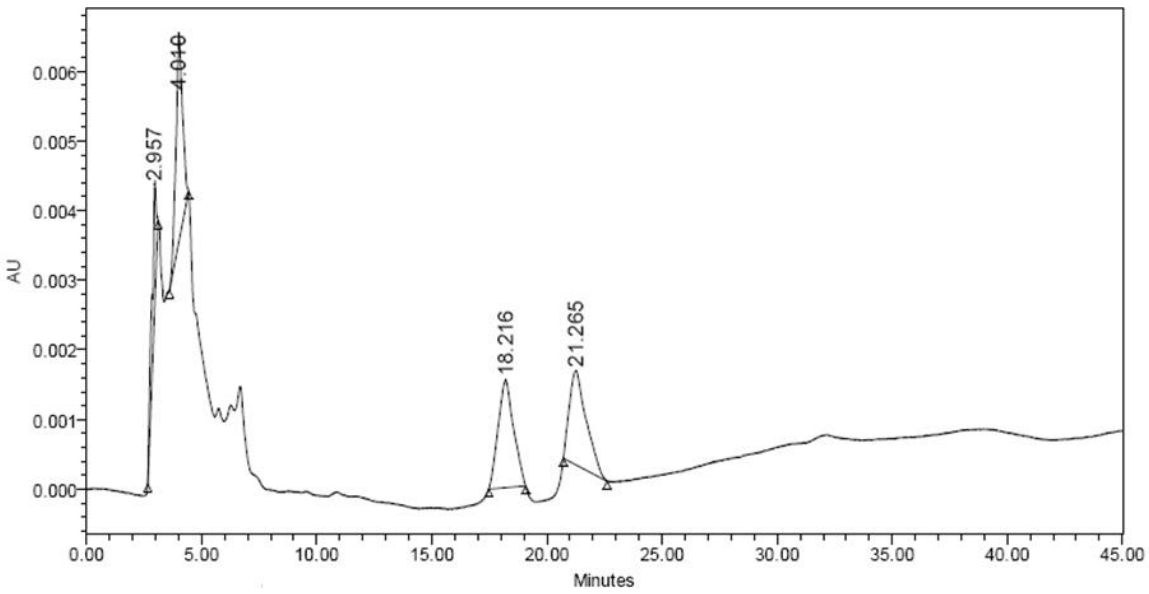
Retention Time 20.628 Represents Psoralen

Fig: 9 Root sample Chromatogram from naturally grown plant



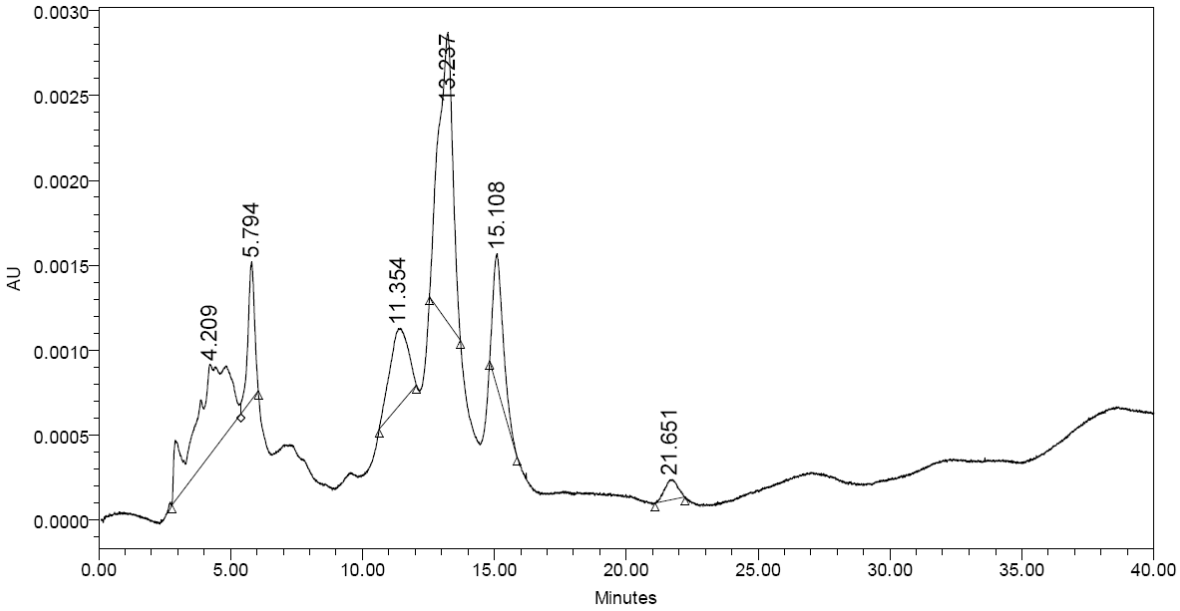
Retention Time 21.312 Represents Psoralen

Fig: 10 HPLC chromatogram of MeJ treated roots



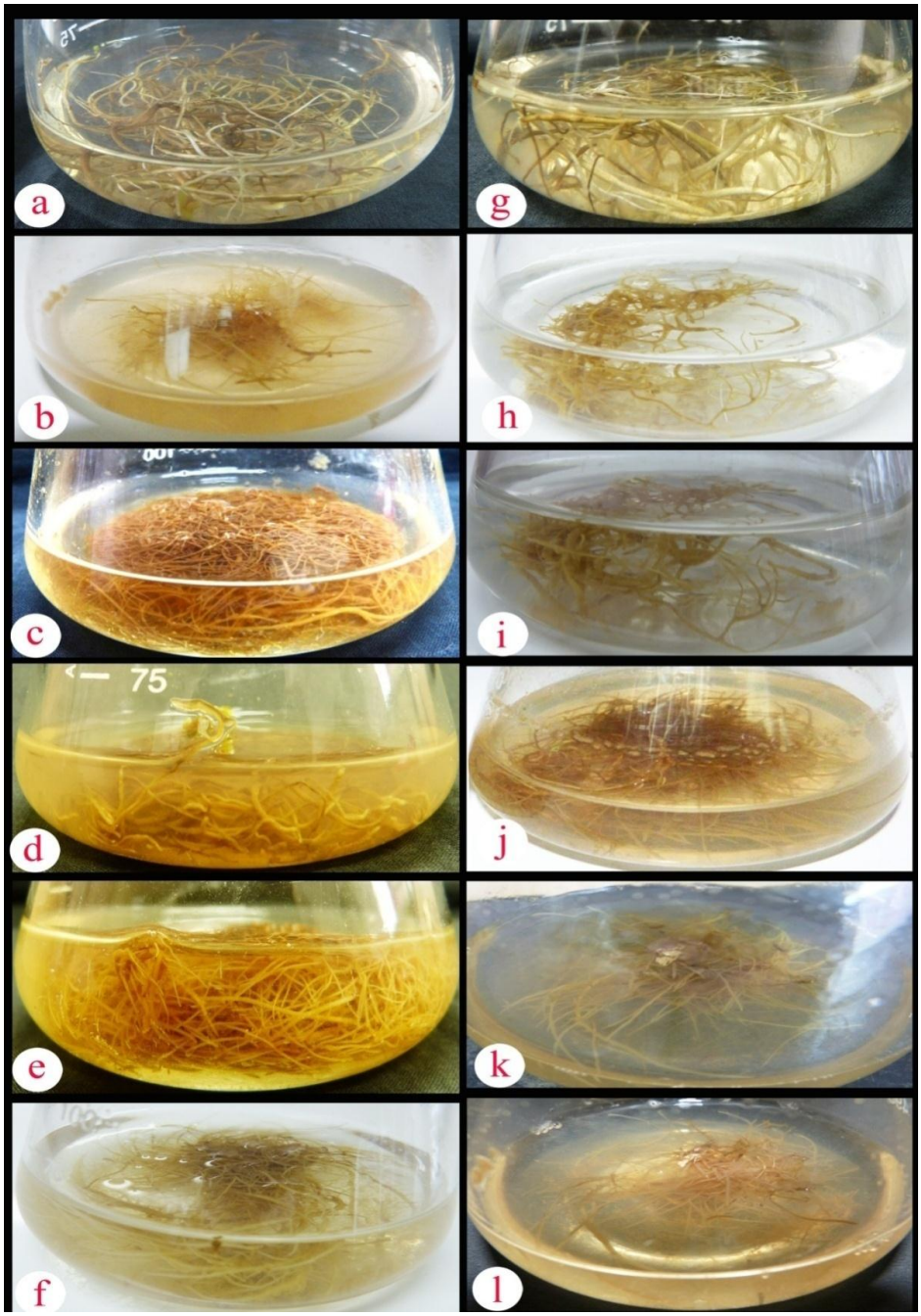
Retention Time 21.265 Represents Psoralen

Fig: 11 HPLC chromatogram of SA treated roots



Retention Time 21.651 Represents Psoralen

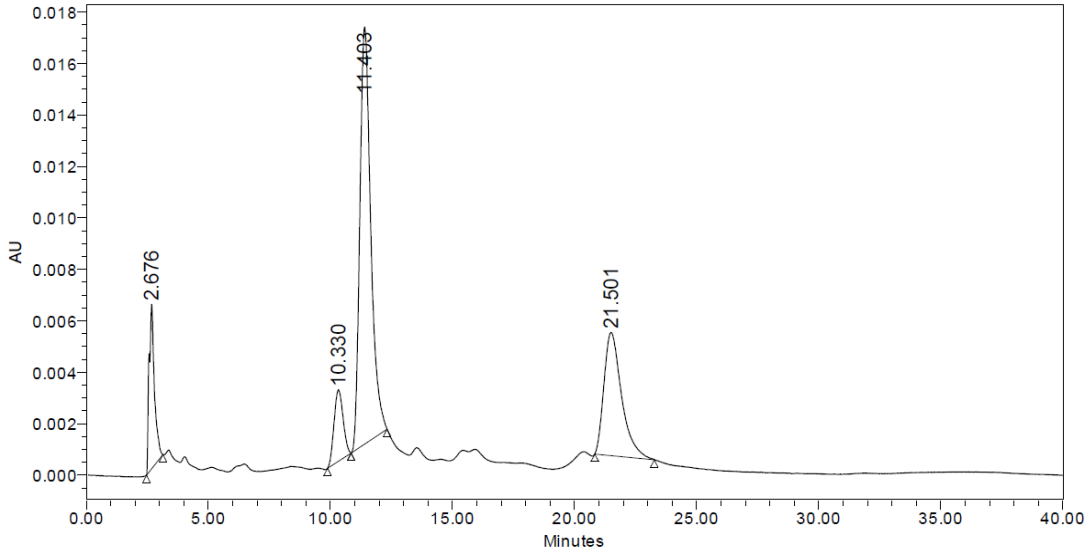
Fig: 12 Effect of different concentration of elicitor treatment (28th day old culture and 8 h contact time) **on *in vitro* adventitious root growth of *Psoralea corylifolia* L.**



a to f - JA treated adventitious root (a. 0µM/L; b. 50µM/L; c. 100µM/L; d.150µM/L; e. 200µM/Land f. 250µM/L)

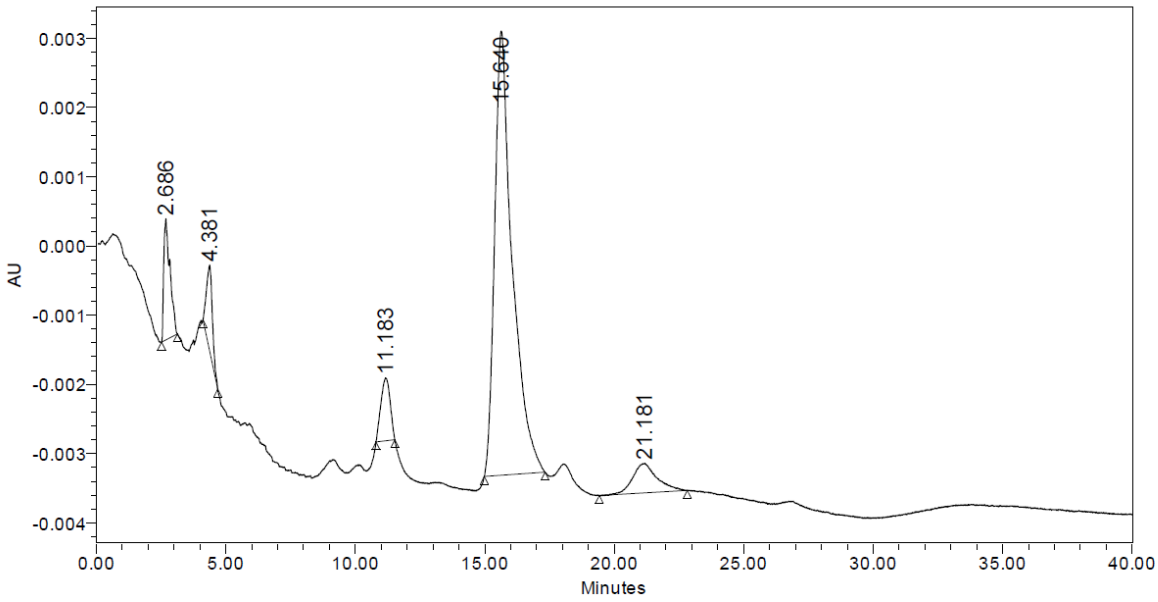
g to l - $AgNO_3$ treated adventitious root (g. 0µM/L; h. 50µM/L; i. 100µM/L; j. 150µM/L; k. 200 µM/Land l. 250µM/L)

Fig: 13 HPLC Chromatogram of JA treated root sample



Retention Time 21.501 Represents Psoralen

Fig: 14 HPLC Chromatogram of $AgNO_3$ Treated root Sample

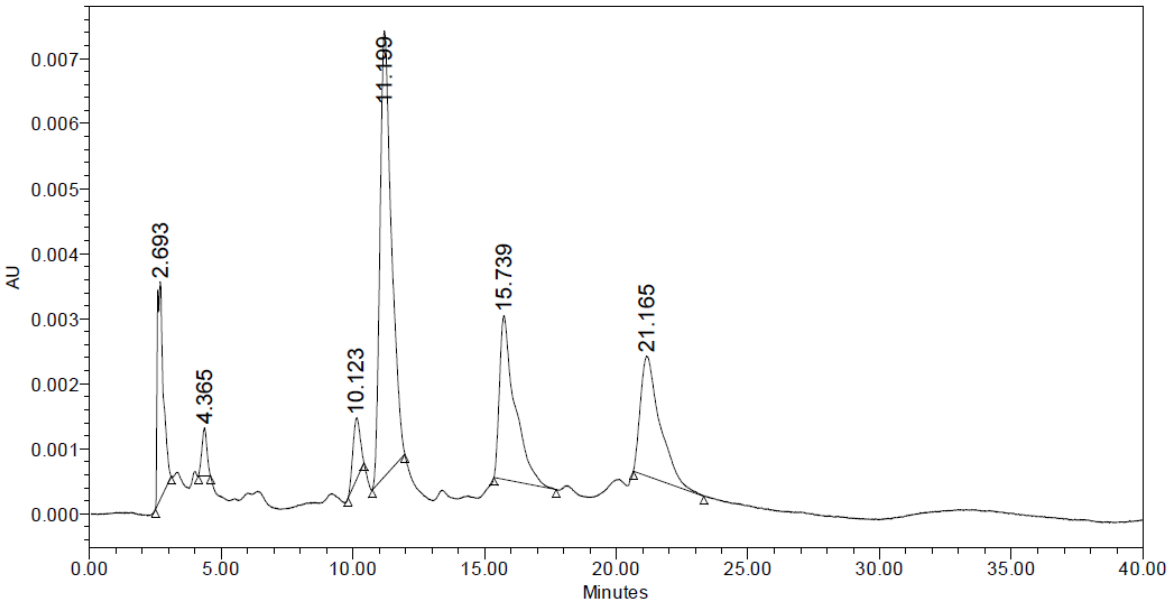


Retention Time 21.181 Represents Psoralen

Figure 15 Hairy root culture of *P. corylifolia* L. in Bubble type Bioreactor

***Agrobacterium rhizogenes*A4 (5L capacity Bubble type bioreactor containing mMS Medium)**

Fig: 16 HPLC profile of psoralen content in hairy root of *P. corylifolia*



Retention Time 21.165 Represents Psoralen

Fig: 17 FTIR analysis of hairy root

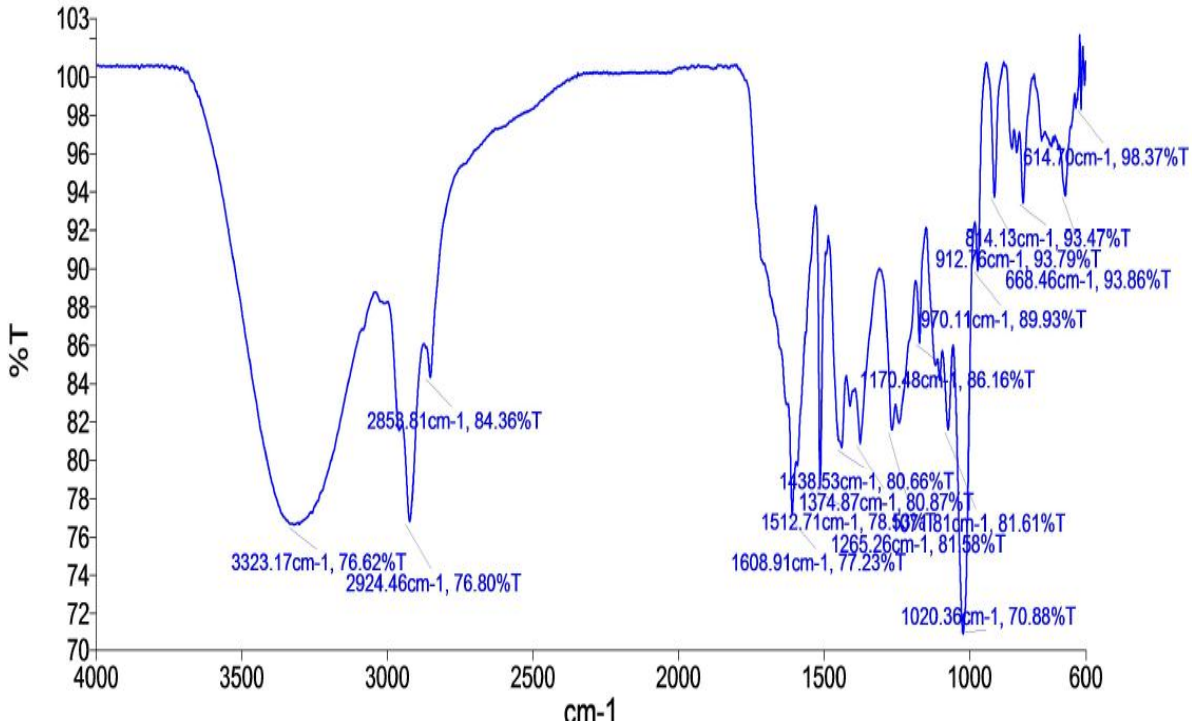
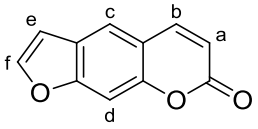


Fig. 18 Nuclear Magnetic Resonance analysis of psoralen in hairy roots of *P. corylifolia*



Psoralen

^{1}H NMR (400 MHz, $CDCl_3$) δ, 6.79-6.85 (m, CH^a, 1H), 6.98-7.09 (m, CH^b, 1H), 7.11-7.14 (m, CH^c, 1H), 7.16-7.19 (m, CH^d, 1H), 7.21-7.30 (m, CH^e & CH^f, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ, 169.77 (C=O), 157.86 (C=C), 144.86 (C=C), 133.28 (C=C), 130.04 (C-H), 128.84 (C-H), 123.75 (C=C), 122.24 (C-H), 116.09 (C-H), 113.29 (C-H), 108.75 (C-H);