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Hairy root induction of *Psoralea corylifolia* for enhanced production of antifungal compound against red rots pathogen *Colletotrichum falcatum*

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***Agrobacterium rhizogenes* strains *viz.,* 532, 2364 and A4 were used to induce hairy roots in *Psoralea corylifolia*. Strain A4 induced the maximum hairy roots in leaf explants with root induction frequency of 80%. Manual wounding of explant with the addition of 100 µM acetosyringone in the medium resulted in better induction of hairy roots. Transfer of Ri plasmid was confirmed by the detection of *ags* gene in transformed hairy roots. Among the different concentrations of hairy root suspension extract tested for antifungal activity, 2.0% concentration displayed 100% inhibition over mycelial growth and spore germination of *Colletotrichum falcatum* at *in vitro* conditions. In addition to that, the extracts were found to be highly compatible with beneficial microflora *viz.*, *Gluconacetobacter diazotrophicus* (endophytic nitrogen fixer in sugarcane), *Bacillus megaterium* (phosphorous solublizer) and *Pseudomonas fluorescens* (biocontrol agent) under *in vitro* conditions. The hairy roots cultured in Erlenmeyer flasks at shaker recorded less multiplication rate due to turbulence of the medium, whereas the hairy roots multiplied in the newly designed airlift bioreactor exhibited 10 fold increases in growth when compared to hairy roots growth in Erlenmeyer flasks which was significantly higher.**

Key words: Hairy roots, *Psoralea corylifolia*, red rot, sugarcane, *Colletotrichum falcatum.*

INTRODUCTION

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic product of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the natural with hope of safety and security. Ethnopharmacologists, botanists, and natural-products chemists are combing the earth for phytochemicals which could be developed for treatment of infectious diseases (Cowan, 1999) (Newman and Cragg, 2012). Botanicals constitute a group of industrially important crops that are of great value for domestic use and export. They are known for rich sources of secondary metabolites such as triterpenes, glycosides, flavonoids, tannins, alkaloids and other aromatic compounds, some of which possess antifungal, antibacterial and antiviral properties apart from other characteristics (Sindhan et al., 1999). Tokin (1960) studied the antibiotic substance produced by higher plants in detail. He proposed the name "Phytoncide" to biologically active substances produced by higher plants

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and studied phytoncide of onion, garlic and some other plants, which contain the strongest antibiotic properties. Secondary metabolites are extracted from plants growing in cultivated fields or in wild stands. The extraction of any metabolite from plants is primarily dependant on two factors; the supply of plants that produces the metabolite and the quantity of the metabolite synthesized by individual plants. To overcome these limitations biotechnologists have suggested hairy root cultures, a new route for enhancing secondary-metabolite production by transformation using the natural vector system possessed by *Agrobacterium rhizogenes*, a Gram-negative bacterium that is the causative agent of hairy root disease in plants (Srinivas Reddy et al., 2012). These fast-growing hairy roots are unique in their genetic and biosynthetic stability, and their fast growth offers an additional advantage to use as a continuous source for the production of valuable secondary metabolites (Ramachandra and Ravishankar, 2002). Hairy root culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. Discoveries of hairy root cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years (San et al., 2007) (Gaviraj and Veeresham, 2011). The major advantages of hairy root culture system over the conventional cultivation of whole plants are, useful compounds can be produced under controlled conditions independent of soil and climatic conditions, cultured cells would be free of microbes and insects, automated control of cell growth and rational regulation of metabolite processes would reduce labour costs and improve productivity, organic substances are easily extractable from callus and hairy root cultures and would yield higher concentration of target metabolites. Due to these advantages, research in the area of tissue or hairy root culture technology for production of plant chemicals has bloomed beyond expectations. Hairy root cultures were also established in various medicinal plants viz., *Tagetus patula* (Kyo et al., 1990), *Lobelia inflate* (Yonemitsu et al., 1990), *Armoracia lapathifolia* (Saito et al., 1991), *Echinacea purpurea*, *Anisodus luridus* (Javanovic et al., 1991), *Swertia japonica* (Ishimaru et al., 1992), *Rauwolfia serpentina* (Benjamin et al., 1994), *Rubia tinctorum* (Ercan et al., 1999), *Artemisia annua* (Archana et al., 2001), *Ocimum basilicum* (Bais et al., 2002) and *Panax ginseng* (Jeong et al., 2003) for the production of medicinal principle compounds. In our earlier studies it was found that 15% aqueous extract of *Psoralea corylifolia* leaves was 100% effective on *Colletotrichum falcatum* under *in vitro* conditions. In continuation of our work, in the present study an attempt was made to produce principle antifungal compounds from hairy root cultures of *Psoralea corylifolia* against *C. falcatum.* The main constraint for commercial exploitation of hairy root cultivation is the development and scaling up of appropriate reactor vessels. In this study an attempt was also made to fabricate a suitable air lift bioreactor for the multiplication of *Psoralea corylifolia* hairy roots.

MATERIALS AND METHODS

Bacterial strains used in this study

A. rhizogenes strains, 532 and 2364 obtained from IMTECH, Chandigarh and strain A4 obtained from Department of Plant Molecular Biology and Biotechnology, TNAU, Coimbatore were used for induction of hairy roots. The bacterial strains were maintained and stored at 4°C on tryptone yeast medium (TY).

Hairy root induction of *P. corylifolia*

P. corylifolia host plants were grown in plain agar medium under *in vitro* conditions. Seeds of *P. corylifolia* were surface sterilized using 0.1% $HgCl_2$ followed by 70% alcohol for 5 min and rinsed with sterilized water for 4 to 5 times. Seeds were then aseptically transferred to tissue culture jar containing sterilized water agar medium and kept for germination. After 10 to 12 days of germination, the leaves and stems were taken out using sterile forceps and excised under aseptic condition and used as explants for hairy root induction. Acetosyringone (ACS), a phenolic compound having chemotactic effects that attract *A. rhizogenes* was used for co-cultivation. ACS at 100 and 150 µM were prepared from 0.1 M stock solution using sterile water. The Murashige and Skoog (MS) medium (Murashige and Skoog,1962) (200 ml) was supplemented with 1 ml of ACS (75, 100, 125 and 150 µM) after autoclaving and cooling of the medium. Then, the medium was thoroughly mixed and poured in Petri plates. The antibiotic, cefatoxime made to a concentration of 1000 mg l^{-1} was filter sterilized in 0.22 µ membrane filter and stored at 4°C. After autoclaving and cooling of the MS medium, antibiotic cefatoxime was added at the rate of 500 mg $L^{-1.}$ A loopful of *A. rhizogenes* strains were inoculated into the 20 ml of sterile TY broth culture tubes and incubated overnight at 25°C at 80 rpm in temperature controlled arbitrary shaker. The explants of *in vitro* propagated *P. corylifolia* plant were aseptically transferred to the sterile filter paper and were cut into 1 to 2 cm bits. Then the explants were pricked with sterile needle and immersed into the overnight grown pelletized *A. rhizogenes* culture suspension for 5 min. The explants were taken out from the suspension and blot dried on the sterile blotting paper. After blotting, the explants were placed into hormone free basal MS medium with ACS at 100 and 150 µM concentrations without antibiotics and incubated in dark at 25°C. After 48 h, the explants were checked for the production of hairy roots and growth of bacteria in the media. When the growth was observed then immediately the explants were transferred to a hormone free MS basal medium containing cefotaxime 500 mg L^{-1}. The explants were transferred to the fresh medium as above till the bacterial growth was completely arrested. The concentration of cefotaxime was reduced in every transfer to fresh medium by 50 mg L^{-1}. After the bacterial free growth was observed, the transformed roots were sub-cultured in hormone and antibiotic free solid basal MS medium. The transformation rate was calculated as follows:

$$\text{Transformation Frequency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A.\ rhizogenes} \times 100$$

Based on the transformation frequency, the explants of host plants were selected. The contaminant free transformed hairy roots were excised from the explants using sterile scalpel blade and transferred to both solid and liquid media, devoid of hormones and antibiotics for subculturing. After subculturing, the plates were kept inverted and maintained in dark at 25°C. Similarly the liquid cultures maintained in

the conical flasks were kept in temperature controlled rotary shaker at 80 rpm in 25°C.

Confirmation of hairy root induction by *A. rhizogenes* strains using *ags (agropine synthase)* gene detection by PCR

The genomic DNA of *A. rhizogenes* strains (532, 2364 and A4) were used as positive control for detection of transformed hairy roots. *A. rhizogenes* cultures were grown for 48 h in TY broth and actively grown cultures were taken for DNA extraction. The genomic DNA from the *A. rhizogenes* strains were isolated using the standard protocol of hexadecy-trimethyl ammonium bromide (CTAB) method given by Melody (1997) with slight modifications. The DNA thus extracted was stored in TE buffer at 20°C for further use. To check the presence of genomic DNA, 10 µl of the samples were loaded in 1.5% agarose gel in 1X TE buffer at 66 volts for 30 to 60 min. The ethidium bromide stained gels were documented using Alpha Imager. Hairy roots were cut into 1 to 2 cm small pieces long and ground in sterilized pestle and mortor containing 300 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM EDTA and 0.5% SDS) and acid-washed sand using a pestle. The homogenate was transferred to a 1.5 ml eppendorf tube and centrifuged at 12,000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant and incubated at -20°C for 20 to 30 min. The crude DNA was pelleted by centrifugation at 12000 rpm for 10 min. Pellets were air dried at room temperature and dissolved in 30 µl of 0.1X TE buffer. PCR was performed to amplify T-DNA *agropine synthase* (*ags*) from the transformed hairy roots.

The genomic DNA of *A. rhizogenes* was used as positive control. Polymerase chain reaction was performed in 25 µl of reaction volume containing 2.0 µl of genomic DNA (*A. rhizogenes* transformed hairy roots), 2.0 µl of 10X PCR buffer containing 1 µM of forward primer (*Ags*-5' GCGCATCCCGAGGCGATG 3') and reverse primer (*Ags*-5'AGGTCTGGCGATCGCGAGGA 3') and 0.15 units of *Taq* DNA polymerase. PCR amplification was performed with a program of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min and stored at 4°C. Agarose gel electrophoresis was performed based on the method given by Sambrook et al. (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. Amplification was verified by electrophoresis on 1.5% agarose gel in 1X TAE buffer using a 1 kb ladder as a molecular weight marker. Gels were stained with ethidium bromide and viewed in UV transilluminater for analyzing the banding pattern. It was then photographed using Alpha imager TM1200 documentation and analysis system.

In vitro* testing of *P. corylifolia* suspension extracts against *C. falcatum

An appropriate amount of standard solution was added to potato dextrose agar (PDA) medium to obtain the desired concentration. Then, temperature of the medium containing suspension extract was raised upto 50 to 55°C in temperature controlled water bath to eliminate the bacterial contamination and poured into petridishes after cooling it to luke warm temperature (Martinez et al., 1990). A mycelial disc (9 mm) of test fungi was taken from 5 to 7 days old culture was placed to the centre of each plate and incubated at 28°C till fungus covered the whole plate in control. After the incubation period, the diameter of mycelial colony was measured and the percent inhibition was calculated by using the formula (Vincent, 1927):

$$\text{Inhibition (\%)} = \frac{(C - T)}{C} \times 100$$

Where C and T were mycelial growth (cm) of test fungi in control and treatments, respectively. All treatments were replicated thrice and average values were taken for interpretation. For checking the antifungal activity of *P. corylifolia* suspension extracts, 0.5, 1.0, 1.5, 2.0 and 2.5% of methanol extracts (approximately 10 g of hairy roots were homogenized in 20 ml of HPLC grade methanol and kept overnight in dark at refrigeration condition. The contents were centrifuged at 4000 rpm for 5 min. The collected supernatant was filtered through Whatman No. 1 filter paper and it was considered as 100% hairy root suspension extract) of suspension were tested against the test pathogen *C. falcatum* by poison food technique. *P. corylifolia* suspension extracts exhibiting effective inhibition percentage were tested at different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5% of methanol extracts) to find out Minimum Inhibitory Concentration (MIC). MIC was defined here as the lowest concentration of the suspension extracts at which no signs of mycelial growth was detectable visually (or) at which maximum antifungal activity was exerted. For checking the effect of suspension extracts of *P. corylifolia* on spore germination of *C. falcatum*, different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5% of methanol extracts of *P. corylifolia* suspension culture) were prepared with sterile water. The conidia from sporulated culture in oatmeal enriched PDA medium were inoculated in extract containing tubes. From each concentration 100 µl was taken into the cavity slides. For each concentration three replications were taken along with the control (without extract). These slides were kept in a moist chamber for the period of 24 h for germination of spores. After the incubation period the spore count taken under microscope. The effect of suspension extract on spore germination was determined and it was compared with the control.

Compatibility of extract against beneficial microorganisms

In vitro testing of the effective suspension extract was carried out on beneficial microflora viz., *Pseudomonas fluorescens, Bacillus megaterium, Gluconacetobacter diazotrophicus* to assess the compatibility based on growth obtained in the respective medium. The 24 h old cultures of beneficial organisms were streaked on the corresponding medium containing effective suspension extract and suitable controls (without extract) were also maintained with 3 replications. The plates were incubated for 2 to 5 days depending on the type of organism and growth of organisms was observed visually compared with that of control and given the remark as – for no growth, + for very less growth, ++ for moderate growth and +++ for good growth equivalent to control. In this experiment, MICs of suspension extracts was used. The beneficial microorganisms were grown on the appropriate media seeded with effective suspension extract to find out whether the organisms retain their original growth rate and growth pattern. Ten milliliter of the corresponding broth without suspension extract of *P. corylifolia* was prepared in a test tube and sterilized in the autoclave at 121°C and 20 lbs pressure for 30 min. The 24 h old culture of a beneficial organism grown on suspension extract containing medium was inoculated in the test tube containing broth and suitable control was also maintained. OD readings were recorded for both the treatments after 48 h at 610 nm so as to distinguish the growth rate. The 24 h old cultures of beneficial organisms grown on medium containing suspension extract of *P. corylifolia* was streaked again on the plates containing medium without suspension extract. The plates were incubated for 2 to 5 days and growth pattern of organism was observed visually and compared with that of control. Growth pattern was defined here as the retaining of the original growth when the organism growing on medium treated with a particular effective suspension extract, was transferred to medium devoid of that extract. It was carried out with an intention to check whether they retain their original morphological characteristics.

Table 1. Selection of culture media for the growth of different strains of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes strains	Liquid medium		
	NB	YEB	TY
532	1	0	2
2364	1	0	2
A4	0	1	2

0, Limited growth; 1, Moderate growth; 2, High growth; NB, nutrient broth; YEB, yeast extract broth; TY, tryptone yeast extract

Table 2. Studies on growth of different strains of *A. rhizogenes* on tryptone yeast extract medium

Time (h)	*Agrobacterium rhizogenes* strains (cfu × 10^5)		
	532	2364	A4
0	0.00	0.00	0.00
3	2.00	3.00	5.00
6	4.00	5.00	7.00
9	5.00	7.00	7.00
12	9.00	11.00	13.00
18	15.00	19.00	19.00
24	18.00	24.00	22.00
36	26.00	28.00	28.00
48	30.00	31.00	44.00
60	32.00	38.00	51.00
72	32.00	37.00	47.00
98	29.00	32.00	41.00
SEd	0.67	0.70	0.95
CD (0.05)	1.39	1.59	1.98

Fabrication of modified airlift bioreactor for mass multiplication of *P. corylifolia* hairy root suspension culture

In view of mass multiplying the hairy root suspension culture in an undisturbed condition, an attempt was made to fabricate a modified airlift bioreactor. The bioreactor was of 3 L capacity, where the working volume was around 2.25 L. The bioreactor was designed using autoclavable borosil glass.

The stand for the bioreactor was made up of 7 kg of stainless steel (rust free) which could hold the whole weight of bioreactor with its full volume. Two provisions were made in the bioreactor, one for air inlet and another for air outlet. Two litres of MS broth was prepared in the bioreactor and autoclaved. Approximately 1 g of hairy roots grown in flasks was inoculated in to the bioreactor in the laminar air flow chamber. A fish tank aerator was used as the source for aerating the bioreactor. The air was filtered through 0.2 µm membrane filter before passing in to the air inlet of bioreactor.

The air was sparged into the media through the air sparger (made of borosil glass with pore size of 1 mm) which was fixed at the bottom of the bioreactor. The air flow was adjusted smoothly such a way that the media inside the bioreactor mixed uniformly without getting any turbulence in the broth. The growth of the hairy root inside the bioreactor was monitored periodically. A provision for air outlet was made on the top of the bioreactor, where another 0.2 µm membrane filter was fixed to arrest outside contamination.

RESULTS

Hairy root induction of *Psoralea corylifolia*

All the 3 strains of *A. rhizogenes* were grown in different culture media *viz.,* NB, YEB and TY, in order to select a common growth medium for all strains. Among the 3 media used, TY broth showed the maximum growth (turbidity) for all the 3 strains (Table 1).

Whereas NB showed moderate growth of 532 and 2364 strains and YEB also showed moderate growth of A4 strain. From these observations, it was considered to use TY medium for further use. The growth of *A. rhizogenes* strains on TY medium was studied and results were presented as colony forming units (Table 2).

All the 3 strains showed maximum growth at 60 h of inoculation. Among the 3 strains, *A. rhizogenes* A4 produced maximum number of colonies (51 × 10^5 cfu) followed by *A. rhizogenes* 2364 (38 × 10^5 cfu) and *A. rhizogenes* 532 (32 × 10^5 cfu). Decline phase starts after 72 h in all the strains. The presence of mega plasmids, responsible for hairy root formation, in *A. rhizogenes* were characterized by in-gel lysis method.

The results revealed that all the *A. rhizogenes* strains had a single mega plasmid with a uniform size. The results confirmed the presence of mega plasmids in *A. rhizogenes* is intact, which will cause hairy roots during transformation.

A study was carried out to screen the best explants for the induction of hairy roots using 3 *A. rhizogenes* strains viz. 532, 2364 and A4.

The co-culturing method as explained earlier was followed for the production of hairy roots using MS medium without growth regulators (hairy root induction does not require any growth regulators). Surface sterilized seeds of *P. corylifolia* were allowed to germinate in water agar and it was used as host plant. From the host plant, leaves, stem and petiole were used as explants for hairy root induction using *A. rhizogenes* strains.

In the study manual wounding, registered lesser percent of hairy roots, whereas when it was combined with ACS addition, hairy root induction frequency was increased. Maximum of 80% of hairy root induction frequency was obtained in case of leaf explants co-cultivated with *A. rhizogenes* strain A4 and with addition of 100 µM ACS, whereas the hairy root induction frequency was decreased when the ACS concentration was increased to 125 and 150 µM. From the results, it was decided to use leaf explants and *A. rhizogenes* strain A4 with addition of 100 µM ACS for further studies (Table 3).

Bacterial free transformed hairy root bits of *P. corylifolia* was subcultured both on solid and liquid tissue culture media without growth regulators. However, a profused growth of hairy roots was observed in liquid media under shaking conditions at 80 rpm.

Table 3. Influence of *A. rhizogenes* strains on hairy root induction frequency of *Psoralea corylifolia* leaf explants in MS medium.

S/N	Treatments	*A. rhizogenes* strains root induction frequency (%)		
		532	2364	A4
1	Control (without *A. rhizogenes*)	0.00	0.00	0.00
2	Manual wounding+ *Agrobacterium rhizogenes*	30.00	20.00	20.00
3	Manual wounding and ACS 75 µM + *A. rhizogenes*	45.00	30.00	40.00
4	Manual wounding and ACS 100 µM + *A. rhizogenes*	60.00	40.00	80.00
5	Manual wounding and ACS 125 µM + *A.rhizogenes*	60.00	40.00	75.00
6	Manual wounding and ACS 150 µM + *A. rhizogenes*	60.00	45.00	60.00
	SEd	1.17	0.81	1.32
	CD(0.05)	2.55	1.76	2.89

ACS – Acetosyringone.

Table 4. Effect of different concentration of hairy root suspension extract of *P. corylifolia* on the growth of *C. falcatum*

S/N	Concentration of suspension extract used (%)	Colony diameter (cm)	Percent inhibition
1	Control	7.0	0.0
2	0.5	5.4	35.6
3	1.0	3.8	56.0
4	1.5	0.8	91.0
5	2.0	0.0	100.0
6	2.5	0.0	100.0
	SEd	0.09	1.80
	CD(0.05)	0.21	3.93

Confirmation of hairy root induction by *A. rhizogenes* strains using *ags* (*agropine synthase*) gene detection by PCR

Molecular analysis of hairy roots was carried out to confirm the transformation of *ags* gene. PCR analysis was done using a pair of gene specific primer which amplified the T-DNA *ags* gene. The total genomic DNA was extracted from *A. rhizogenes* and resolved in agarose gel electrophoresis. It showed that the DNA was intact and the concentration was nearly uniform in all the isolates of *A. rhizogenes*, which can be further used as positive control for PCR analysis. The total genomic DNA was extracted from the transformed hairy roots to observe the presence of DNA. It showed that the DNA was intact and the concentration was nearly uniform in all the samples transformed by different *A. rhizogenes* strains. These DNA samples were subjected to PCR amplification. In PCR, the primer showed amplification which confirmed the successful transformation of T-DNA *ags* gene which produced the band size of 341 bp (Plate 1).

In vitro testing of *P. corylifolia* suspension extracts against *C. falcatum*

The hairy root suspension culture of *P. corylifolia* was developed in the Erlenmeyer flasks. Standardization of extraction protocol was done for suspension extracts so as to have maximum recovery of specific antimicrobial compounds which was determined in terms of maximum antifungal activity shown by the suspension extracts prepared in a given solvent. As the compound of interest, psoralen is intracellular, methanol showed effective extraction of the compounds from suspension extract. The suspension extract of *P. corylifolia* was subjected for evaluation at different concentrations so as to find out the minimum inhibitory concentration against the growth of *C. falcatum*. To check the antifungal activity of suspension extracts, 0.5, 1.0, 1.5, 2.0 and 2.5% of suspension extracts were tested against mycelial growth of *C. falcatum* and the results are presented in Table 4. The results showed that minimum inhibitory concentration was 2.0%, where it showed 100% inhibition over mycelial growth of *C. falcatum* under *in vitro* conditions (Plate 2).Whereas in our earlier studies, leaf extracts of *P. corylifolia* used at 15% concentration exerted a complete inhibition of mycelial growth and spore germination of *C. falcatum.* This result clearly indicates the presence of higher concentration of desired principle antimicrobial compound in the suspension extract than that of the leaf extract. Available reports on *P. corylifolia* suggest that the antifungal activity has been tested only with the plant parts. But the present study confirmed that antifungal

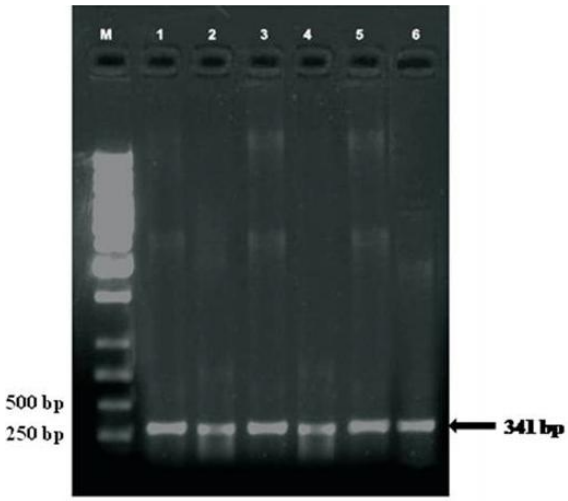


Plate 1. Confirmation of hairy root induction by *A. rhizogenes* using *ags* gene detection by PCR. M - 1 kb ladder, 1, *A. rhizogenes* 532 genomic DNA; 2, *A. rhizogenes* 2364 genomic DNA; 3, *A. rhizogenes* A4 genomic DNA; 4, *A. rhizogenes532 mediated P. corylifolia hairy root; 5, A. rhizogenes 2364 mediated P. corylifolia hairy root; 6, A. rhizogenes A4 mediated P. corylifolia hairy root.*

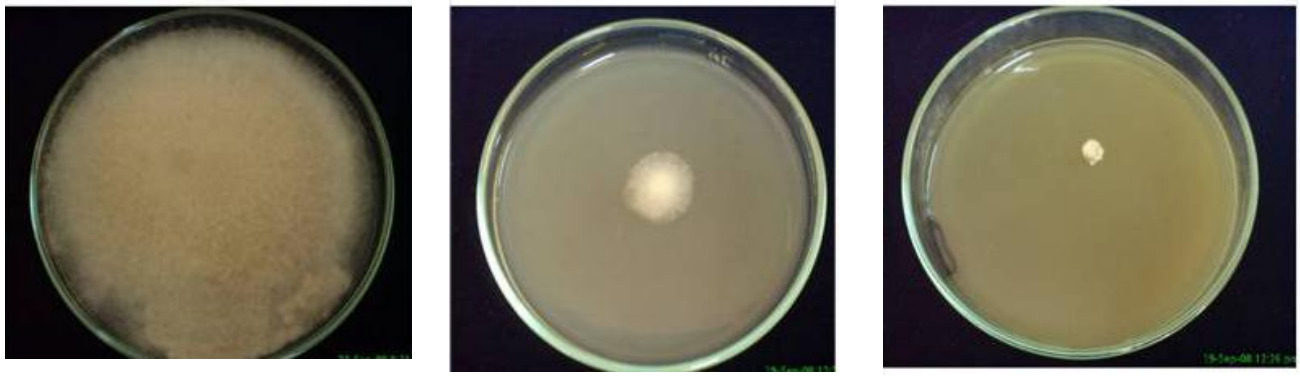


Plate 2. Effect of suspension extracts of *P. corylifolia* on the growth of *C. falcatum.*

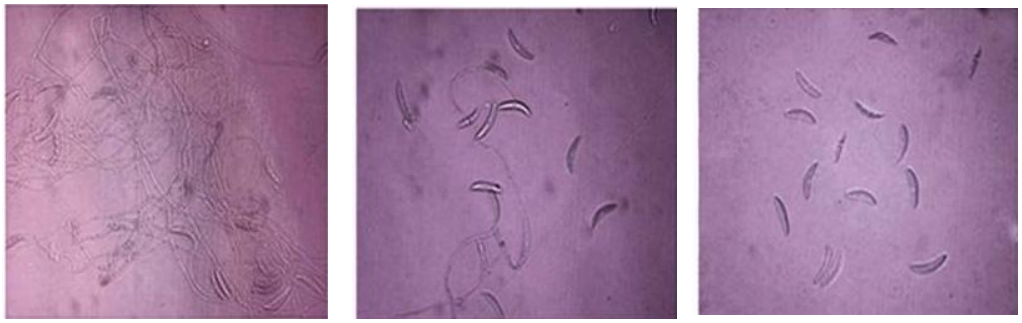
principles are also present in suspension cultures of *P. corylifolia,* and it was also clear that the concentration of principle antifungal compounds in the suspension extract was at higher level when compared to plant extracts. The effective suspension extract of *P. corylifolia* which exhibited the inhibition on the mycelial growth of *C. falcatum* were subjected to check its effectiveness on spore germination of *C. falcatum.* Different concentrations of suspension extracts (0.5, 1.0, 1.5 and 2.0%) of *P. corylifolia* were prepared and the spores were inoculated in the extract containing glass vials. Among 4 different concentrations checked 2.0% showed 100% inhibition against the spore germination followed by 1.5% which showed 84.2% inhibition (Table 5) (Plate 3).

Compatibility studies of effective suspension extracts of *Psoralea corylifolia* versus beneficial microflora

The effective concentration (2.0%) of suspension extract was taken for testing the compatibility assessment with *P. fluorescens, B.* m*egaterium, and G. diazotrophicus*. The suspension extracts showed no inhibitory effect on beneficial microflora and found to be fully compatible with all beneficial microflora. Colorimetric testing was done to check whether there is any deviation in the growth rate of beneficial organisms which have been grown on the media treated with selected concentrations of the suspension extracts and they were compared with their

Table 5. Evaluation of effective suspension extract on conidiospore germination of *Colletotrichum falcatum* under *in vitro* conditions

S/N	Concentration of suspension extract used	Number of spores germinated/ microscopic field	Percent inhibition
1	Control	19	0.0
2	0.5%	14	26.3
3	1.0%	8	57.8
4	1.5%	3	84.2
5	2.0%	0	100.0
	SEd	0.27	1.59
	CD(0.05)	0.61	3.55



Control **1.5%** **2.0%**

Plate 3. Effect of suspension extract of *P. corylifolia* on conidia germination of *C. falcatum*.

Table 6. Effect of effective suspension extract of *P. corylifolia* on the growth rate of beneficial microorganisms.

S/N	Name of the organism	Colorimetric reading of cultures treated with effective suspension extracts of *P. corylifolia* at 610 nm (after 48 h)					SEd	CD
		Percent						
		0.5	1.0	1.5	2.0	Control		
1	*Bacillus megatherium*	0.28	0.25	0.27	0.26	0.27	0.01	0.02
2	*Pseudomonas fluorescens*	0.42	0.46	0.41	0.46	0.45	0.02	0.04
3	*Glucanoacetobacter diazotrophicus*	0.46	0.40	0.39	0.43	0.43	0.02	0.04

growth in the respective suspension extracts free media. However, no such deviation was reported. All the colorimetric readings were found to be on par with each other. The plating tests conducted also did not show any change in growth pattern of the tested organisms (Table 6).

Multiplication of *P. corylifolia* hairy roots in modified airlift bioreactor

Earlier in the study *P. corylifolia* hairy root suspension culture was first multiplied in 250 ml Erylnmeyor flasks under shaken condition at 80 rpm. Development of hairy root was attempted several times. There was no proper multiplication of hairy roots in the Erlenmeyer flasks containing suspension medium (MS broth devoid of growth regulators) under shaken conditions. The shaking condition might have disturbed the hairy root proliferation. To overcome the disturbed condition of turbulence an alternate method was considered to mass multiply the compound for which an attempt was made to fabricate a modified airlift bioreactor. Two litres of MS broth prepared in the bioreactor was inoculated with approximately 1 g of hairy roots. The growth of the hairy roots inside the bioreactor was monitored periodically. A significant growth of hairy roots was observed after first week of inoculation onwards. There was no contamination observed throughout the experimental period of 30 days, and also faster multiplication of hairy roots was observed. After 30 days from inoculation, the hairy roots were harvested and its wet weight was taken. Ten grams of hairy roots was harvested from the bioreactor at the end of the experiment which was inoculated with 1 g of hairy

roots. The growth rate of hairy roots was found to be higher in the airlift bioreactor when compared with hairy roots grown in Erylnmeyor flasks, where only 3 g of hairy roots was obtained at the end of 30 days incubation.

DISCUSSION

Hairy root induction of *P. corylifolia*

Agrobacterium rhizogenes strains *viz*. 532, 2364 and A4 were cultured on nutrient agar (NA) modified broth, yeast extract (YE) broth and tryptone yeast extract (TY) broth for selecting a suitable medium. Tryptone yeast extract was found to be the best culture medium among the 3 tested media. Among the media used, the composition of TY medium alone has tryptone. TY medium supports a good growth of *Rhizobium* without over production of polysaccharides.

To get precise results through molecular studies, a medium which supports lesser polysaccharide production are only preferred. In several studies TY medium was only used to culture *Rhizobium* (Balachandar et al., 2003). Since *Agrobacterium* comes under the family Rhizobiaceae, TY medium might have supported the ideal growth of *A. rhizogenes* compared to other media tested.

White and Nester (1980) reported that *A. rhizogenes* 1583 strains contain one megaplasmid responsible for the development of hairy root disease. To align with, gel lysis method employed in the present study also displayed to contain a mega plasmid in all the 3 tested *A. rhizogenes* strains.

Moreover gel lysis is a suitable method to visualize such large sized (› 250 kb) plasmids (Balachandar et al., 2003).These megaplasmids with uniform size are present in all the three cultures and this study confirmed the virulence of *A. rhizogenes* strains for inducing hairy roots.

To test the hairy root production of *P. corylifolia*, 3 different *A. rhizogenes* strains were used on MS basal medium by manual wounding and with different concentrations of acetosyringone (ACS) treatment. Among the 3 explants used (leaf, stem and petiole), leaf explants co- cultured with *A. rhizogenes* strain A4 showed the higher induction of hairy root.

Profused root growth characterized by the formation of numerous lower order branches has been obtained. The formation of lower roots is essential for rapid increase in root biomass and the establishment of continuous cultures (Fortin et al., 2002). *Daucus carota* L. and *Convolvulus sepium* L. were among the earliest species to be transformed using *A. rhizogenes* conn. (Tepfer and Tempe, 1981). *In vitro* host plant of *P. corylifolia* was studied for the optimized production of hairy roots at 75, 100, 125 and 150 µM of acetosyringone (ACS) using MS medium.

Leaf, stem and petiole were used as explants from the host. The study revealed that leaf explants produced the maximum induction of hairy roots when compared to stem and petiole. The efficiency of explants is known to differ with different explants sources. The hairy root induction frequency was found to be higher in leaf explants of *Coleus forskohlii,* when compared to other explants (Aswin, 2006).Results of the present study showed the production of hairy roots with the manual wounding in all the 3 strains and media used. The manual wounding along with ACS (100 µM) treatment resulted in higher frequency of hairy roots than manual wounding alone and also the time taken for hairy root induction was reduced by 2 weeks compared to the application of manual wounding. Wounding is a prerequisite for the genetic transformation process through *Agrobacterium* and might help in the production of signal phenolics (Gelvin, 2000) and enhances the accessibility of putative cell- wall binding factors to the bacterium. ACS is one such compound used successfully to enhance transformation in various plant species through *A. rhizogenes* mediated genetic transformation (Gelvin, 2000). Acetosyringone and α-hydroxy acetosyringone induce the transcription of *vir* region (The *vir* region on the Ti/Ri plasmid is a collection of genes whose collective function is to excise the T-DNA region of the plasmid and promote its transfer and integration into the plant genome). Bacterial cultures and explants co-cultivated on MS basal medium with 100 µM ACS addition reduced the time of hairy root induction by a week compared to explants infected with *A. rhizogenes* without *vir* gene induction (Archana et al., 2001). Sonification with 100 µM ACS treatment was found to be the best (86%) for enhancing the transformation frequency of hairy root induction in tobacco than 150 µM ACS (Kumar et al., 2006). Similar observation is obtained in the present study, where 100 µM of ACS treated manually wounded explants induced hairy roots with the maximum frequency of 80%. ACS has also known to enhance the transformation efficiency due to activation of *ags* gene in *A. rhizogenes* (Aoki et al., 1997). Therefore it was presumed that the results obtained in the present investigation corroborates with the earlier investigation and that the enhancement in transformation by ACS treatment might be due to the activation of *ags* gene absolutely responsible for the delivery of T-DNA to plant tissues. The present experiment showed that, the best root formation response (as transformation frequency) was achieved with *A. rhizogenes* A4 strain which induced the maximum of 80 per cent hairy roots at 100 µM ACS. Efficiency of transformation is known to differ with different bacterial cultures. The transformation ability of different *A. rhizogenes* strains was found to be different in tobacco, where the performance of *A. rhizogenes* strains was reported to be in the order of LBA 9402, 9340, 9365, 15834 and A4 (Archana et al., 2001).

Confirmation of hairy root induction by *A. rhizogenes* strains using *ags* (agropine synthase) gene detection by PCR

For a long time, the *Agrobacterium rhizogenes ags g*ene has been considered to be modulator of plant growth and

cell differentiation. A new function of the *ags* gene in plant–*Agrobacterium* interaction became apparent with the discovery that the gene was a potential activator of secondary metabolism in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae, Papillionaceae and Rosaceae families. In some cases, the activator effect of *ags* gene is sufficient to overcome the inability of cultured plant cells to produce large amounts of secondary metabolites. The integration of Ri T-DNA leads to alterations in hormone metabolisms, transport properties and the production of opines (low molecular weight compounds found in plant crown gall tumors produced by the parasitic bacterium *Agrobacterium*) in the transformed roots. Ri T-DNA consists of 2 parts T_L-DNA and T_R-DNA which is physically separated by a non-transformed DNA sequence of approximately 18 kb (Jouanin, 1984) the T_R-DNA. *A. rhizogenes* mediated T-DNA transfer in plants is thought to be a random process. The T_L / T_R DNA integration in host genome might lead to alterations in the morphology and physiology of the roots. Detailed investigations on *A. rhizogenes* mediated Ri T-DNA transfer in plant genome would reveal a better understanding of the importance of T_L-DNA / T_R DNA genes in the induction of hairy roots. Molecular analysis of *P. corylifolia* hairy roots through PCR was done using a pair of primer, which amplified the T- DNA *agropine synthase* gene and confirmed the transformation at 341 bp segment. Similar primer was also used by Gartland et al. (2001) in *Ulmus procera* to confirm the transformation. The confirmation of transformation by detection of opines was also reported in *Artemisia annua* L. (Archana et al., 2001). Based on the integration of Ri T_L-DNA and T_R-DNA genes, the transformed root clones were characterized into 3 groups (Batra et al., 2004) using southern hybridization. The studies also demonstrated the role of T_L-DNA and T_R-DNA genes on growth and alkaloid accumulation in the root clones.

In vitro testing of *P. corylifolia* suspension extracts against *C. falcatum*

Different concentrations of suspension extract were subjected for evaluation to find out the minimum inhibitory concentration against the growth of *C. falcatum*. Among the different concentrations (0.2, 1.0, 1.5 and 2.0%), the complete inhibition of mycelial growth and spore germination was observed at 2.0% suspension extract of *P. corylifolia* under *in vitro* conditions. Whereas in the earlier studies of Jeyakumar (2007), it was reported that only 15 per cent aqueous extract of *P. corylifolia* leaves exerted a complete inhibition of mycelial growth and spore germination of *C. falcatum.* This result clearly indicates the presence of higher concentration of desired principle antimicrobial compound in the suspension extract than that of the leaf extract. The genetically transformed root cultures produce high levels of secondary metabolites compared to those of intact plants (Srivastava and Srivastava, 2007). The antifungal activities of *P. corylifolia* are already known. Rajendra et al. (2004) reported the antifungal activity of extracts obtained from *P. corylifolia* seeds against dermatophytic fungi such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum gypseum*. Mazzuca et al. (2003) also reported that the antifungal activity of its seed extract against fungi causing infection in humans.

Biri singh (2004) screened 18 medicinal plants against different plant pathogens and reported that 10% aqueous extract of *P. corylifolia* was completely effective against the fungal pathogen, *C. falcatum.* Available reports on *P. corylifolia* suggest that the antifungal activity has been tested only with the plant parts. But the present study confirmed that antifungal principles are also present in suspension cultures of *P. corylifolia,* and it was also clear that the concentration of principle antifungal compounds in the suspension extract was at higher level when compared to plant extracts. On comparing the effect of cell suspension extract and hairy root extract, both extracts exhibited 100% inhibition over *C. falcatum* at 2% concentration. But the time taken for establishment of cell suspension was longer when compared to hairy root development, where even growth hormones are also not required. Because of these advantages of hairy root suspension culture over cell suspension culture, it was decided to go for hairy root suspension cultures for production of antimicrobial compounds from *P. corylifolia.*

Compatibility studies of effective suspension extracts of *P. corylifolia* versus beneficial microflora

The present study was not only carried out with an aim to find out fungitoxic potential of suspension extracts, but also to check whether these extracts were compatible to the existing beneficial rhizosphere and phyllosphere microbes often used as biofertilizers and antagonists. After finding out the effective concentration of suspension extract (2% showing a good deal of fungitoxic activity under *in vitro* conditions), it was subjected to check for compatibility with the beneficial microflora. The effective suspension extract tested, was reported fully compatible with the beneficial microflora taken up for the study. The results in the present study were not in confirmation with that of Parimala and Sabitha (2000) who examined the compatibility of six plant extracts *viz*., *Azadirachta indica*, *Abrus precatorious*, *Vitex negundo*, *Nerium oleander* and *Catharanthus roseus* (toxic to *Pheisariopsis personata* and *Puccinia arachidis* causing late leaf spot and rust in groundnut respectively) for *Trichoderma viride* strains (MNT 5 and MG 6), *P. fluorescens* and *Bacillus subtilis*, but found these plant extracts were incompatible to the tested antagonists. This might be due to the reason that the tested plant extracts might be possessing different type of compounds which are of inhibitory nature to the beneficial antagonists. Biri Singh (2004) reported that the aqueous extracts of *P. corylifolia* (which was inhibitory to *Colletetrichum falcatum,* red rot causing fungi) showed

100% compatability with beneficial microorganisms *viz Bacillus megatherium*, *P. fluorescens* and *Gluconacetobacter diazotrophicus*, which was also confirmed by the studies of Jeyakumar (2007). Literature on compatibility testing of suspension extracts with beneficial microflora viz., Rhizobium sp., Azospirillum lipoferum, Azotobacter sp., Bacillus megaterium, Gluconacetobacter diazotrophicus, Burkholderia vietanamiensis, P. fluorescens and T. viride had not been reported earlier.

Multiplication of hairy roots in modified airlift bioreactor

The main constraint for commercial exploitation of hairy root cultivation is the development and scaling up of appropriate reactor vessels (bioreactors) for the delicate and sensitive plant hairy roots. In the present study, an attempt was made to design a bioreactor that would support a good growth and multiplication of hairy roots of *P. corylifolia,* possessing the antifungal compound in larger quantity. The hairy roots inoculated in the newly designed modified airlift bioreactor (3 L capacity) showed a better growth (1 g hairy root inoculum yielded 10 g of its wet weight after 30 days of incubation) when compared to the growth of hairy roots in Erlenmeyer flasks (1 g hairy root inoculum yielded only 3 g of its wet weight). The result clearly indicates that the growths of hairy roots were influenced by the bioreactor. The reason for faster multiplication might be due to supply of air through external source (aerator) and also may be due to uniform agitation of the medium without much hindrance to the growth of hairy roots. This was the initial attempt made to develop a special type of bioreactor and fruitful results were obtained. This pioneer work will pave way for further studies, where larger bioreactors can be designed with the control systems, in such a way that the multiplication rate of hairy roots can further be increased.

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