

## Full Length Research Paper

# Establishment of hairy root culture and production of secondary metabolites in *Coleus* (*Coleus forskohlii*)

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**Production of hairy roots through transformation and *in vitro* culture was attempted in *Coleus* using MTCC 2364 strain of *Agrobacterium rhizogenes*. Nodal stem part and mature leaf were used as explants. The nodal stem part responded with huge amount of root production. From the nodal part first 3 to 4cm long shoot emergence took place and after that within 12 days roots started emerging from base of node. If the shoot portion was cut and transferred to new MS basal medium it started producing root within 5 days. The confirmation of transformation of plant was done by polymerase chain reaction (PCR) with *rolA* gene primer. It showed 400 bp band. The hairy root recorded the highest amount of forskolin in comparison with any other plant part.**

**Key words:** *Coleus*, hairy roots, transformation, primer, *Agrobacterium rhizogenes*.

## INTRODUCTION

*Coleus* (*Coleus forskohlii* Briq.) is an important medicinal plant. Roots of this plant contain an alkaloid called forskolin which has high pharmaceutical value. *Coleus* is grown as a field crop to harvest the roots for forskolin extraction. Separate land area is required for this purpose. Biotechnological interventions will be helpful to produce the active principle *in vitro* in the laboratories. Hairy roots of *Coleus* induced as a result of genetic transformation by *Agrobacterium rhizogenes* has been reported to produce forskolin (Sasaki et al., 1998). Hairy roots of many species have been reported to produce large variety of secondary metabolites similar to plant from which it is derived and they maintain the stability of growth through the successive generation (Mukundan and Hjorsto, 1990). Ikenaga and Muranaka (1999) initiated hairy roots in *Solanum aculeatissimum* by using *A. rhizogenes* strain AICC 15834 and insertion of T-DNA was confirmed by polymerase chain reaction (PCR)

analysis which amplified *rolB* gene. The present investigation was carried out to initiate and establish hairy root culture and assess the accumulation of forskolin in *Coleus*.

## MATERIALS AND METHODS

### Hairy root culture

*A. rhizogenes* strain, MTCC 2364 was used for induction of hairy roots. The bacterial strain MTCC 2364 was maintained at 4°C on solid Nutrient Agar Medium (NAM). A single colony of *A. rhizogenes* was taken from culture grown on NAM medium stored at 4°C. It was inoculated on to the 20 ml of yeast extract broth (YEB) (Vervliet et al., 1975) medium in a sterile conical flask. The inoculated cultures were grown for overnight at 28°C in arbitrary shaker at 200 rpm. The cultures were ensured to have 0.6 to 0.8 optical density (OD) at 600 nm.

The mature leaves and nodal part of stem were taken after

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proper surface sterilization. After trimming the margins, leaves were cut into 0.5 cm<sup>2</sup> size bits and the stem were cut longitudinally into two halves. Some explants were pinched with needle, some were soaked and some were pricked and soaked both. Later, the explants were immersed into the overnight grown *A. rhizogenes* culture suspension for 15 min.

The explants were taken out from the suspension and blotted dry on the sterile blotting paper. After blotting, the explants were placed into the co-cultivation medium in dark for 24, 48 and 72 h. MS medium (Murashige and Skoog, 1962) was used for hairy root culture of *C. forskohlii*. MS medium supplemented with sugar (3%) was used for co-cultivation.

After co-cultivation, the leaves with petioles were inoculated onto the hormone free MS and B5 basal medium containing cefotaxime 500 mg/L to check the bacterial growth. The cultures were maintained at 25°C with 16/8 h light and dark period.

## Molecular analysis of hairy roots

### Genomic DNA extraction from hairy roots for PCR

Hairy roots were cut into the small pieces of 1 to 2 cm long and ground in a 1.5 ml Eppendorf tube 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 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 (1 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0). For each PCR reaction, 1 µl (50 to 100 ng) of this DNA preparation was used as template.

### Isolation of genomic DNA from *Agrobacterium rhizogenes*

The genomic DNA of *A. rhizogenes* was used as positive control for detection of transformed hairy roots. The genomic DNA from the *A. rhizogenes* was isolated using the standard protocol of hexadecyltrimethyl ammonium bromide (CTAB). *A. rhizogenes* culture was grown for 48 h in TY broth. Actively grown culture of 30 ml quantity was taken in a centrifuge tube and was centrifuged at 6,000 rpm for 5 min at 4°C. When the supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells. Again, when centrifuged at 6000 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of butanol. Again, the pellet was resuspended in 2 ml TE buffer added to 100 µl lysozyme (10 mg/ml freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10% SDS and 25 µl of 100 µg/ml proteinase K were added, mixed well and incubated at 37°C for 1 h. To this, 200 µl of 5 M NaCl was added and mixed well. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65°C for 10 min. The mixture was extracted with 1 ml of phenol: chloroform mixture, mixed well and centrifuged at 600 rpm for 15 min at 4°C. The aqueous layer was transferred carefully to a 2.0 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated 1 h to overnight at -20°C. The DNA was pelleted by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One microlitre DNase free RNase (10 mg/ml) was also added by swirling and incubated at 37°C for 30 min. The DNA was stored at -20°C for further use. For each PCR reaction, 2.0 µl of this DNA preparation was used as template.

## PCR analysis

PCR was performed to amplify *rolA* gene. Reactions were performed in a final volume of 20 µl. The mixture contained 50 to 100 ng of genomic DNA, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 10 mM of each of dNTPs, 5 mM of upstream and downstream primers and 0.15 units of *Taq* DNA polymerase.

The primer sequences used are RolAF: 5' GGAATTAGCCGGACTAAACG 3' and RolAR: 5' AGGTCTGGCGATCGCGAGGA 3'. 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. PCR amplified products (20 µl) were subjected to electrophoresis in a 1.5% agarose gel in 1X TBE buffer at 100 v for 2.0 h using Apex submarine electrophoresis unit. The ethidium bromide stained gels were documented using Alpha Imager TM 1200 – Documentation and Analysis system of the Alpha Innotech Corporation, USA.

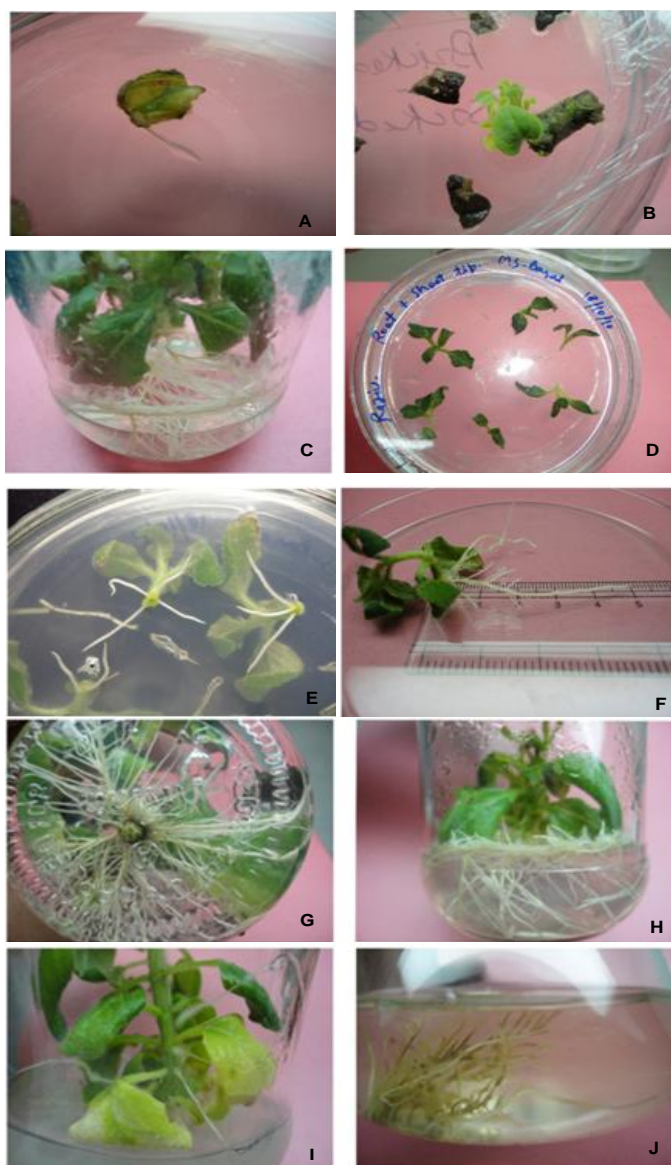
Forskolin content in the hairy root was assessed using High Performance Chromatographic System equipped with LC8A pump, SPD-M 10A vp photo array detector in combination with class LC 10A software.

## RESULTS AND DISCUSSION

Hairy root culture of *C. forskohlii* by using leaf and shoot as explant was obtained by co-cultivation with *A. rhizogenes* strain number MTCC 2364. The transformation frequency was up to 30%. For leaf, the best co-cultivation time ranged from 24 to 48 h, but for stem it was 72 h. For leaf, the induction of root occurred in 8 days, but for stem it was 12 days. The maximum transformation frequency (30%) was obtained in those explants which were pricked and soaked both during co-cultivation followed by pricked one (Table 1). It may be because of more chance of *A. rhizogenes* to get in contact with injured part of explants. The leaf and internodal part of stem started producing hairy roots from the pricked or wounded sites. A few stem explants first showed regeneration from nodal part and after 18 days, they started producing root. These types of plants were totally transformed that was confirmed by PCR analysis with *rolA* gene primer. If the upper twig of these types of plant was cut and transferred in new MS basal media, then within 5 days they started producing huge amount of roots. Even from shoot portion also, roots started growing (Figure 1). *A. rhizogenes*, a soil pathogen can infect wounds of plant and induces hairy roots in a number of plant species. Hairy root cultures established by transformation with *A. rhizogenes* are attractive system for the production of plant secondary metabolites, because of its independence of seasonal and geographical conditions, biochemical and genetic stability, rapid growth rates, and ability to produce secondary metabolites at levels comparable to the mother plants (Christey and Braun, 2005; Srivastava and Srivastava, 2007). Molecular analysis of hairy roots was done to confirm the transformation. PCR analysis was done using pairs of gene specific primers

**Table 1.** Study of co-cultivation of different explants of *Coleus forskohlii* with *Agrobacterium rhizogenes* strain MTCC 2364.

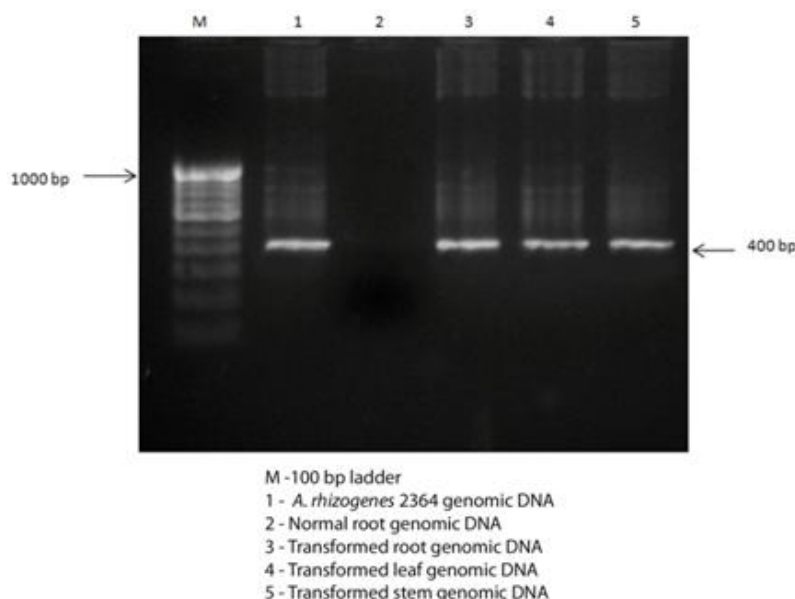
S/N	Co-cultivation time (h)	Leaf explants response (%)			Shoot explants response (%)		
		Pricked	Soaked	Pricked+Soaked	Pricked	Soaked	Pricked+Soaked
1	24	20	2	30	0	0	0
2	48	10	Explants died	20	0	0	5
3	72	Explants died	Explants died	Explants died	5	10	30
4	96	Explants died	Explants died	Explants died	Explants died	Explants died	Explants died



**Figure 1.** Hairy root culture in *Coleus forskohlii*: (A) After co-cultivation root emerged from explant; (B) After co-cultivation from nodal part of stem shoot started emerging; (C) After 25 days it produced lots of root; (D) Upper shoot portion was cut and transferred; (E) Within 5 days from the base of shoot, root started emerging; (F) Measured length of root after 10 days of inoculation of cut shoot; (G) Transferred shoot started producing root in huge amount in MS basal medium; (H) Amount of root started increasing day by day; (I) Shoot portion also started producing root; (J) Established hairy root culture in B5 medium.

**Table 2.** HPLC forskolin analysis in hairy roots of *Coleus forskohlii*.

Hairy root culture and <i>in vitro</i> plants	Amount of forskolin present in dry weight of sample (mg/g)
Root	2.36
Established root	1.16
Leaf	0.853
Stem	0.420

**Figure 2.** Confirmation of hairy root induction by *A. rhizogenes* using *rol A* gene detection by PCR.

(forward and reverse) which amplify the T-DNA *rolA* gene, present in *A. rhizogenes*. The primers showed amplification which confirmed the successful transformation. T-DNA *rolA* gene produced band of size 400 bp (Figure 2).

After four weeks of culture, tips of hairy roots were cut down and inoculated on to the MS and B<sub>5</sub> liquid and semi solid medium without any growth regulators and with cefotaxime 400 mg/L. The cultures were maintained at 25°C in dark and the roots in liquid medium were maintained on rotary shaker at 100 rpm. The cultured tips showed axillary growth within three weeks of culture and these actively grown root tips were again sub cultured at regular interval of three weeks. Totally, four subcultures were done. The concentration of cefotaxime was reduced in every subculture to 200 mg/L. The induced hairy roots were sub cultured on MS and B<sub>5</sub> liquid medium for further proliferation. The proliferation of hairy roots was seen on B<sub>5</sub> liquid medium (Figure 1).

By this method, we can produce large amount of hairy roots in short duration in comparison to any other method. Even from shoot, portion roots started emerging. These events show that whole plant becomes transformed with *Agrobacterium* genes. Gartland et al. (2001) conducted

molecular analysis of hairy roots through PCR, using two pairs of primers, one to amplify T DNA *agropine synthase* gene and another for the *rolA* gene and confirmed the transformation in *Ulmus procera*. In this work, only *rolA* gene primer was used. The confirmation of transformation by detection of opine was also reported in *C. forskohlii* (Sasaki et al., 1998; Wei et al., 2003, 2005). The hairy root culture was also established using B<sub>5</sub> basal medium. There is a report by Li et al. (2005) that hairy root culture of *C. forskohlii* established in B<sub>5</sub> medium showed the highest amount of rosmarinic acid in comparison with any other medium.

High performance liquid chromatography (HPLC) analysis revealed good amount of forskolin accumulation in hairy roots (Table 2). It is concluded that hairy root culture is a potential option for forskolin production in *Coleus*.

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