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*Full Length Research Paper*

# Hairy Root Culture of *Eclipta Alba (L.)* Hassk.

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## ABSTRACT

*Eclipta alba* (L.) Hassk. was transformed by *Agrobacterium rhizogens*. Explants from healthy growing roots were inoculated with *A.rhizogens* strain MTCC 532. The genetic markers included vir D1 and rol B, responsible for T-DNA transfer and hairy root initiation. Bacterial gene transfer was confirmed using these markers. The rol B genes were detected using PCR analysis.

**Key words:** *Eclipta alba*, *Agrobacterium rhizogens*, MTCC 532, PCR, Hairy root induction

## 1. INTRODUCTION

Various natural products like tropane alkaloids, atropine, hyoscyamine, scopolamine and steroidal precursors are mainly produced by roots (Kumar and Roy, 2006; Kumar and Sopory, 2008 and Kumar and Shekhawat, 2009). Cell cultures have been established from many plants and they have been used for the production of secondary metabolites. Often they do not produce sufficient amounts of the required secondary metabolites (Rao and Ravishanker, 2002). Cell cultures have been used to achieve only a few commercial processes inspite of considerable efforts. In some cases, secondary metabolites are only produced in organ cultures such as hairy root or shooty teratoma (tumor-like) cultures. For example, hairy roots produce high levels of alkaloids (Sevo'n and Oksman-Caldentey, 2002). A large number of efficient regeneration and *Agrobacterium* mediated transformation protocol have been developed for different medicinal plants, which in turn could be used for the production of valuable secondary metabolites.

*Agrobacterium rhizogenes*, a soil plant pathogenic bacterium, possesses a plasmid called the Ri (root-inducing) plasmid. There is an increased production of various phenolic compounds when plants are wounded. This, in turn, enhances the expression of the virulence (*vir*) genes on the bacterium's Ri plasmid, by inducing acetosyringone. The *vir* genes encode enzymes that enable the bacteria to insert a well defined DNA fragment, the T-DNA of its Ri-plasmid into the genome of plant cell around the contact site (Gelvin, 2000). This T-DNA encodes enzyme that regulates the production of two groups of compounds, the plant growth hormones

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(i.e., auxins and cytokinins) and unusual amino acids, opine. The plant growth hormones cause the transformed plant cells to form 'hairy' roots, while the opine serve as an exclusive food source for *A. rhizogenes* (Lehninger *et al*., 1993). Hairy root cultures have high growth rate and are able to synthesize root derived secondary metabolites.

In the early 1980's, the formation of hairy roots in several dicotyledonous plants by *A.rhizogenes* was reported by Chilton *et al.* (1982). The production of secondary metabolites form *in vitro* transformed roots of many plant species have been reported, for example, higher levels of pulchelin E produced from *Rudbeckia hirta* (Luczkiewicz *et al*., 2002), plumbagin from *Plumbago zeylanica* (Verma *et al*., 2002), high scopolamine from *Hyoscyamus muticus* (Zolala *et al*., 2007), silymarin production from *Silybum marianum* (L.) Gaertn (Rahnama *et al*., 2008), production of alizarin and purpurin in *Rubia akane* Nakai (Lee *et al*., 2010) and tropane alkaloids production in hairy root cultures of *Atropa belladonna* (Yang *et al*., 2011).

Cell cultures tend to produce low yields of secondary metabolites. In contrast, transformed hairy roots grow rapidly and produce higher levels of secondary metabolites than the parent plants. Therefore, they can serve as a good alternative source of phytochemicals (Hughes and Shanks, 2002; Kumar and Sopory, 2010; Bopana and Saxena, 2010 and Kumar and Roy, 2011). A significant increase in growth of whole culture or specific aspects of the cultures and in the production of secondary compounds from transformed hairy roots have been reported by many researchers (Dhingra *et al*., 2000). An increased level of secondary metabolites in the hairy roots of *Datura stramonium* (Maldonado-Mendoza *et al*., 1993) and *Tropaeolum majus* (Wielanek and Urbank, 1999) as compared to whole plants has been reported. Different elicitors such as chitosan, methyl jasmonate, heavy metals and salicylic acid (DiCosmo and Misawa, 1985; Barz *et al*., 1988; Gundlach *et al*., 1992; Ebel and Cosio 1994 and Poulev *et al.*, 2003) have been used to increase the production of secondary metabolites. Enhancement of *Agrobacterium tumefaciens* mediated transformation efficiency by pre treatment and wounding of plant tissue provides improved technologies (Hraska and Rakousky, 2011).

Secondary metabolites derived from metabolically engineered plants have a high potential. For example, changes in flower color or increased level of antioxidative flavonol production in tomato has been achieved by modifying anthocyanin and flavonoid pathways. (Bourgaud *et al*., 2001 and Kirsi-Marja and Inze, 2004). However, to date, only a few pharmaceutically important compounds have been produced by successfully modifying their pathways. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems (Roessner *et al*., 2001). However, hairy roots, transformed with *Agrobacterium rhizogenes*, have been found to be more suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions.

## 2. MATERIALS AND METHODS

(a) Explant preparations:

Healthy growing roots from *Eclipta alba* were used as explants for hairy root induction. These were sterilized by following standard procedures.

(b) Organism used:
The strain of *Agrobacterium rhizogenes* used in the present study - MTCC 532.
(c) Bacterial culture medium:
Inoculation of the bacteria in yeast extract broth (YEB) culture medium was used to obtain suspension of *Agrobacterium rhizogenes* (MTCC 532) and this suspension was left standing at 250 rpm for 16 hr at $25^0$C in an orbital shaker under constant stirring. Subsequently the bacterial suspension was centrifuged at 5000 rpm for 10 min. after transferring to a sterilized centrifuge tube. The residue obtained was then suspended in liquid MS media supplemented with 3% sucrose.
(d) Hairy root culture medium:
MS medium (Table-3.1) supplemented with 0.8% agar, 3.0% (w/v) sucrose and pH of 5.7 was maintained and used as growth medium for induction of hairy roots from explants.
(e) Establishment of hairy root culture:
The surface sterilized explants were cut below epicotyl and then fine pointed needles were used for puncture inocluating the epicotyl 1.5 cm above the cut with MTCC 532 strain of *A. rhizogenes*. The explants were co-cultivated with 25 ml *A. rhizogenes* in liquid MS media in 100 ml flasks at 200 rpm in a shaker under dark. The root explants were then blotted on sterile tissue paper after incubation period of 3 hr and were placed on the growing medium containing cefotaxime (250 mg/l) (to eliminate excess bacteria). Half of inoculated explants were incubated in light (16 hr light/8 hr dark) and the other half in dark at 24 ± $2^0$C. Punctured controls without *A. rhizogenes* treatment were also planted under each condition. The obtained roots were then excised and cultivated individually in MS culture medium in the presence of cefotaxime (250 mg/l) for total elimination of *Agrobacterium rhizogenes*. Transformed roots, cultivated in MS culture medium, were kept in the dark at 24 ± $2^0$C and at intervals of 21 days they were subcultured.
(f) PCR analysis:
The two genes, *rol* B and *vir* D1 play a prominent role in transformation. DNA isolated from *A.rhizogenes* strain, transformed hairy root and control were used for PCR amplification (Corbett research).
(i) DNA isolation:
3g of each sample was homogenized in absolute alcohol. The homogenized material was handled as per the method described by Doyle and Doyle (1990) in following steps:
1. 4g of tissue material was grounded in absolute alcohol with the help of mortar-pestle.
2. The homogenized material was transferred to 20 ml pre-warmed ($60^0$C) DNA isolation buffer (2X CTAB DNA extraction buffer - 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 2 µl/ml β-mercaptoethanol) in capped polypropylene tubes.
3. Clump was suspended by using spatula.
4. Incubated for 1 hr. at $60^0$C with occasional mixing by gentle swirling in water bath.
5. After removing from water bath one volume of chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for 15 min to ensure emulsification of the phases.
6. Spun at 15,000 rpm for 15 min (Eltec centrifuge)
7. Aqueous phase was taken and transferred to another tube.
8. Ice cold 2 vol of absolute alcohol or 0.6 vol. of isopropanol was added to precipitate DNA.
9. DNA-CTAB complex was precipitated as a fibrous network, lifted

by Pasteur pipette and was transferred to washing solution. In some cases amorphous precipitation was collected by the centrifugation at 5,000-10,000 rpm for 5-10 min at $20^0$C.

10. 20 ml of 70% alcohol was added to the pellet of DNA and was kept for 20 min with gentle agitation.
11. The pellet was collected by centrifugation at 5,000 rpm for 5 min at $20^0$C.
12. The tubes were inverted and drained on a paper towel. The pellet was dried over-night after covering with parafilm with tiny pores.
13. The pellet was re-dissolved in 1 ml of TE buffer by keeping over night at $4^0$C without agitation.

(ii) Purification of DNA:

RNA was removed by treating the sample with DNase free RNase procured from Pure-gene, USA. Protein including RNase was removed by treating with chloroform: Isoamyl alcohol (24:1). The purification was carried out in following steps:

1. 2.5 µl of RNase was added to 0.5 ml of crude, DNA preparation (2.5 µl of RNase = 25 µg of RNase, so treatment was 50 µg / ml of DNA preparation).
2. Gently it was mixed thoroughly and was incubated at $37^0$C for 1 hr.
3. After 1 hr, a mixture of 0.3 - 0.4 ml of chloroform: Isoamyl alcohol (24:1) was added and mixed thoroughly for 15 min till an emulsion was formed.
4. Spun for 15 min at 15,000 rpm.
5. Supernatant was taken avoiding the whitish layer at interface.
6. The DNA was re-precipitated by adding double the quantity of absolute alcohol.
7. To pellet the DNA, the tube was centrifuged for 5 min at 5,000-10,000 rpm.
8. The pellet was washed with 70% alcohol and dried over night.
9. The DNA was re-dissolved in 500µl of TE buffer.

(iii) Gel analysis:

The integrity of DNA was judged through gel analysis in following steps:

1. Cast 150 ml agarose gel (0.8%) in 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 µg /ml of Ethidium bromide.
2. 2 µl of DNA per sample was loaded in each well.
3. Known amount of uncut Lambda phage DNA was also loaded as control.
4. Electrophoresis was conducted at 50 V for 1 hr.
5. Gel was visualized under UV light using transilluminator.
6. Presence of single compact band at the corresponding position to λ phage DNA indicates high molecular weight of isolated DNA.

(iv) Quantitation of DNA:

The quantitation of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a UV-VIS spectrophotometer (Mecasys Company Ltd., made in Korea) in following steps:

1. 200 µl TE. buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelengths.
2. Added 4 µl of DNA, mixed properly and record the absorbance (A) at both 260 and 280 nm.
3. DNA concentration was estimated by employing the following formula:

$$DNA\ (\mu g/\mu l) = \frac{A260\ x\ 50\ x\ dilution\ factor}{1000}$$

4. Quality of DNA judged from the ratio of A values recorded at 260 and 280 nm.

(v) Dilution of DNA for PCR:

The quantitated DNA was diluted to final concentration of 25 ng/µl in TE

buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) for PCR amplification.

(vi) PCR:

The *rol* B and *vir* D1primers were supplied by Chronos Biotech., Delhi. The 5' primer sequence of *rol* B gene was TGGATCCCAAATTGCTATT CCTTCCACGA and 3' primer sequence was TTAGGCTT CTTTCTTCAGGTTTACTGCAGC. A minimum of 780 base pair (bp) fragment could be detected in PCR amplification. For *vir* D1 gene, the 5' primer sequence ATGTCGCAAGGACGTAAGCCCA and 3' primer sequence GGAGTCTTTCAGCATGGAGCAA were used. This amplified the DNA fragment of 450 bp from the DNA samples. For checking the presence of *rol* B gene, the PCR amplification was done in a final reaction volume of 30 µL containing 1 PCR buffer (Bangalore Genei), 1.5 mM $MgCl_2$, 1 mM each of the four dNTPs, 1.25 U of Taq polymerase (Bangalore Genei) and 0.5 mM each of 5' and 3' primers with 3 µl of the total DNA from transformed roots. After initial denaturation at $94^0$C for 3 min, PCR was performed for 35 cycles at $94^0$C for 30 sec, $55^0$C for 30 sec and $72^0$C for 1 min followed by a final extension at $72^0$C for 7 min. The same conditions were also used for the detection of *vir* D1 gene.

Following the amplification, the PCR products were loaded on 1.2% agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing 0.5 µg/ml of the Ethidium Bromide. The amplified products were electrophoresed for 3-3.5 hr at 100 V with cooling. After separation the gel was viewed under UV trans-illuminator and photographed by digital camera.

## 3. RESULTS AND DISCUSSION

The hairy roots of *E. alba* obtained were white, slender, highly branched with several lateral branches (Plate-4.9-A).

Hairy root cultures were identified both by morphological as well as genetic markers. The important morphological markers included rapid growth, lateral branching and plagiotropism (negatively geotropic). The genetic markers included *vir* D1 and *rol* B, responsible for T-DNA transfer and hairy root initiation. Bacterial gene transfer was confirmed using these markers. The *rol* B genes were detected from the transformed root DNA using PCR analysis. The *vir* D1 genes were not found in the hairy root DNA and this confirmed the absence of *Agrobacterium* residual genes in the transformed tissues. In control, both the genes were absent (Plate-4.9-B).

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