

TLC Method for the Determination of Plumbagin in Hairy Root Culture of *Plumbago rosea* L.

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Abstract: Multiple shoot formation from the medicinal plant *Plumbago rosea* Linn. was induced on Shoots from nodal segments on Murashige and Skoog media containing cytokinin. BAP (2.0 mg lG¹) added to the media gave best Shoot induction, while Kin (2 mg lG¹) induced shoot formation. Four bacterial strains, A4, TR105, LBA 9402 and 15834 and the one explant type, *in vitro* developed shoots were examined for hairy root induction. Among the *Agrobacterium rhizogenes* strains used, 15834 strain was observed to be the most virulent one. The establishment of hairy root cultures leading to the production of a bioactive compound, Plumbagin and it was confirmed by using TLC-densitometric method.

Key words: *Plumbago rosea* % Hairy root induction % *Agrobacterium rhizogenes* 15834 strain % PCR and TLC

INTRODUCTION

Plumbago rosea L. (Plumbaginaceae) is a medicinal shrub distributed throughout India. The tuberous roots of *P. rosea* are used as an important indigenous ayurvedic drug for treating a variety of symptoms including oedema, piles, rheumatism, secondary syphilis and leprosy. The medicinal property of the roots are attributed to the presence of plumbagin, 5-hydroxy 2-methyl 1,4-naphthoquinone. Plumbagin has many pharmacological properties of which anticancerous and antimicrobial activities are the most important [1], it also exhibits action against HIV-1 [2]. Therefore the plant needed immediate attention to sort out alternative means of its conservation.

Hairy root formed after insertion of the T-DNA of the *Agrobacterium rhizogenes* [3] are capable of regeneration, show rapid growth, are capable of producing the secondary metabolites of the mother plant and also useful in germplasm conservation [4]. In nature, *P. rosea* does not set seeds and hence shoot cuttings are used for vegetative propagation. Being a root drug, *P. rosea* have to be destructively harvested in nature over generations and consequently natural population of the species seldom occurs. Keeping in mind the importance of conservation of this plant, this paper describes regeneration as well as transformation protocols and Plumbagin determination for *P. rosea*.

MATERIALS AND METHODS

The nodal explants of *P. rosea* from one-year-old plants were collected from the medicinal plant Garden of the JJ College, Pudukkottai, Tamil nadu, India. Young nodes from newly sprouted young stems (2-3 weeks old) of *P. rosea* were cut and washed in distilled water. They were washed with 5% teepol for 8-10 mins and then it was washed with 70% ethanol followed by 0.1% (W/V) mercuric chloride for a few seconds and thoroughly rinsed three-four times in sterilized distilled water. About 1.0 cm long sprout segments cultured on agar solidified MS medium supplemented with various concentration of BAP for induction and proliferation of multiple shoots. pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained under cool-white fluorescent light (2,000 lux) at 25±2°C with 16hrs photoperiod.

Wild strains of *Agrobacterium rhizogenes* A4, TR105, LBA 9402 and 15834 were grown on YEM medium for 48 hrs and were used for infection. Aseptically developed shoots were freshly pricked with a sterile needle. The wounded shoots were then immersed in the resuspended *Agrobacterium rhizogenes* suspension for different period of time, blotted dry and cocultured for three days in the dark on MS medium with 1 mg/ l BAP. The shoots were immediately sub-cultured on the 250 mg ml¹ cefotaxime containing MS medium when

bacterial growth was seen on the media. After obtaining bacteria free cultures, they were subcultured on MS solid medium without antibiotics.

The transformation was confirmed by PCR analysis by the CTAB procedure given by Doyle and Doyle [5]. Genomic DNA was extracted from both hairy roots and untransformed roots (control) of *P. rosea*. Plasmid DNA was extracted from strains A4, TR105, LBA 9402 and 15834 using the SDS/alkalinelysis method [6]. PCR analyses were run by using each DNA sample as the template, to test for the presence of the rol B in the T-DNA. Primers were designed according to the DNA sequence of the rol B gene (862 bp) described by Slightom *et al.* [7] and Jouanin [8], respectively. The primers for amplifying the rol B gene were 50-TACTGCAGCAGGCTTCATGCA-30 and 50-GCTTTCCCAGACCAGAGACTG-30. Each PCR mixture consisted of approximately 200 ng plant genomic DNA (or 100 pg plasmid DNA), 0.4 mM of each primer, 0.2 mM dNTPs, 1.0 unit Taq polymerase and 2.5 ml 10_ PCR buffer in a final volume of 25 ml. PCR was run in the following conditions: initial denaturation at 94 8C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were examined by electrophoresis on 1.2% (w/v) agarose gel.

Plumbagin was extracted with chloroform from dried hairy roots by using the methodology described by Hebel *et al.* [9] and Crouch *et al.* [10]. Hairy roots were harvested, air-dried, powdered and extracted with chloroform for 12 hrs. Plumbagin content was determined using a TLC-densitometric method [11]. Silica gel 60 F254 was used as a stationary phase and toluene: glacial acetic

acid (99:1) was used as the mobile phase. The solvent system allowed a clear separation of plumbagin from other extract constituents. The co-chromatographed authentic sample of plumbagin (Sigma Chemicals Co., USA) was used to detect the presence of plumbagin on the TLC plate after the run.

RESULTS AND DISCUSSION

Nodal segments were cultured on MS medium supplemented with different concentrations and combinations of BAP and Kin. The best response was obtained when the explants were cultured on MS medium containing 2.00 mgL⁻¹ BAP. In this concentration, 4-6 were developed from each explants and this number was found to be increase (8.2 ± 1.2) following the maintenance of these cultures in same medium from this type of explants (Fig. 1 and Plate 1A). Similar results were already reported for many plumbaginaceae plants such as, *Plumbago zeylanica* [12-14] and *Plumbago rosea* [15].

For hairy root induction, after 7 days of infection, small protuberance emerged at the wounded sites of aseptic shoots infected with the bacteria and subsequently these structures differentiated into white cottony hairy roots in another period of 15 days. There were no hairy roots formed from control. These *A. rhizogenes* strains were not equally efficient in hairy root induction. Strain 15834 conferred the highest transformation rates on aseptic shoots examined (7.82 ± 0.31). This is followed, in order, by strain TR105 (4.21 ± 0.26) and strain LBA9402 (3.02 ± 0.11) (Fig. 2). These results were similar to the observation on *Rubia tinctorum* [16] and *Arachis hypogaea* [17].

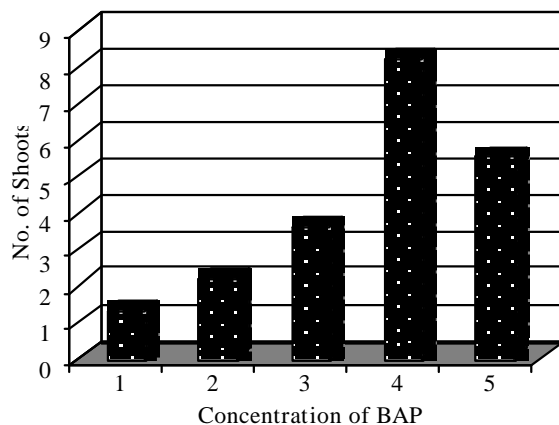


Fig. 1: Effect of BAP on shoot induction

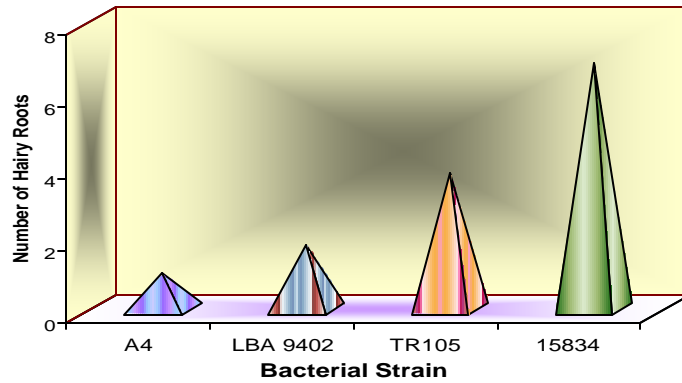
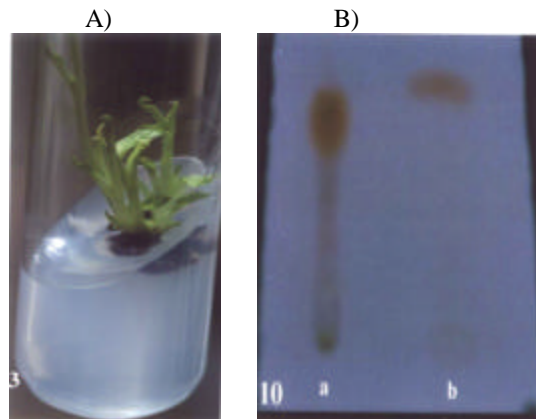


Fig. 2: Hairy root induction in *Plumbago rosea* L. by different strains of *Agrobacterium rhizogenes*.



A) Multiple shoots on MS + 2.00 mgL⁻¹ BAP
a) Standard Marker

B) TLC of Plumbagin from hairy roots
b) Plumbagin from hairy root culture of 15834.

Plate 1: Multiple shoots of *Plumbago rosea* L. and TLC of Plumbagin

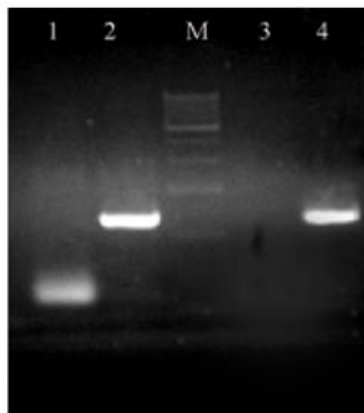


Plate 2: PCR analysis of rolB gene from *Agrobacterium rhizogenes* 15834 strain.

- 1: rolB specific PCR product from the transformed plant
- 2: Rubisco specific PCR product (~600 pb) from the transformed plant M: 500 bp DNA ladder
- 3: rolB specific PCR product from the untransformed plant
- 4: Rubisco specific PCR product (~600 pb) from the untransformed plant

A. rhizogenes-mediated transformation was confirmed by PCR analysis. By using primers designed according to the sequence of the rol B in the TL-DNA and the PCR was successfully amplified rol B gene from the genomes of hairy root and not in DNA isolated from the untransformed or transformed roots (Plate 3). Both transformed and untransformed roots of *Plumbago rosea* had been subcultured in 1/2 MS liquid medium and plumbagin was identified from these roots by using TLC-densitometric method. Both the standard and the sample were run in toluene:glacial acetic acid solvent system and the bright yellow spot corresponding to authentic sample was identified as a plumbagin (Plate 1b).

CONCLUSION

The use of *Agrobacterium rhizogenes* is a popular method for gene transfer in plants because of its ability to transfer the Ri plasmid into the host plant via t-DNA. As a result with strain *Agrobacterium rhizogenes* 15834 was found to be an effective means of inducing hairy root formation of *Plumbago rosea* (L.). The hairy cultures showed fast growth rates. Most importantly, the establishment of hairy root cultures leading to the production of a bioactive compound, Plumbagin.

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