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Development of hairy root culture system of *Phlogacanthus thyrsiflorus* Nees

Rajani Singh[a], Swati Kamal[a], Deepa Rani[a], Kunal Mukhopadhyay[b], Madhuparna Banerjee[a,*]

[a] College of Biotechnology, Birsa Agricultural University, Ranchi 834006, Jharkhand, India
[b] Department of Biotechnology, Birla Institute of Technology, Mesra, Ranchi 835215, Jharkhand, India

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ABSTRACT

Hairy root cultures were established from leaves, shoot tips and node explants of *in vitro* grown plants upon co-cultivation with *Agrobacterium rhizogenes* Conn (strain ATCC 15834) by transformation. Hairy root induction was observed after 7 d on all three explants co-cultured for different periods. Amongst different durations of co-culture (45, 75, 105, 135, 165, 195, 225 and 255 min), 225 min was found to be the best for all the three explants. Shoot tip explants showed maximum percentage of root induction (93.33%) as well as highest number of roots (4.80) per explant. PCR analysis of *rolC*, and *aux1* genes was performed in transformed and non-transformed roots and confirmed. Specific amplification products of 815 bp and 487 bp were deserved in transformed roots by *aux1* and *rolC* primers.

1. Introduction

Transgenic hairy root culture has revolutionized the role of plant tissue culture in secondary metabolite production. They are unique in their genetic and biosynthetic stability, faster in growth, and can be maintained more easily. Using this methodology a wide range of chemical compounds has been synthesized (Giri and Narasu, 2000; Shanks and Morgan, 1999). Advances in tissue culture, combined with improvement in genetic engineering, specifically transformation technology, have opened new avenues for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances (Hansen and Wright, 1999). *Phlogacanthus thyrsiflorus* Nees, commonly known as *Ram Vasak*, belongs to the family Acanthaceae growing mostly during December–April in north east region of India. The whole plant is extensively used for its great medicinal value. Ethnobotanical experiences as well as clinical test revealed medicinal importance of this plant. Leaf juice is used in cough, phlegm, asthma, bronchial disorders, jaundice, diarrhoea, dysentery, tuberculosis, malarial fever, and rheumatism. Plant extract also exhibits analgesic activity (Mukherjee et al., 2009) and antibacterial activity (Singh and Singh, 2010). Flowers are antidote to pox, prevent skin diseases like sore, scabies, *etc.* and used as vegetables. Medicinal salt extracted from the ash of whole plant is used to cure indigestion, gastritis, pharyngitis, cough, and asthma. The paste of root is used in case of chronic leucorrhoea. The plant has got anti-allergic property. The smoke of leaf is used during asthma attack in the form of a cigarette (Kalita and Bora, 2008; Patwari, 1992).

Though, a lot of work has been done on induction of transformed hairy roots from various plants of different genera of this family, there is no report on induction and establishments of hairy roots in *P. thyrsiflorus*. Considering the importance of this valued but comparatively unexplored medicinal plant, the present work has been conceived to explore its susceptibility to *Agrobacterium*

* Corresponding author. Tel.: +91 9430330220.
E-mail address: madhuparna8@gmail.com (M. Banerjee).

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rhizogenes for induction of hairy roots and its genetic evaluation, so that its implication on acceleration of useful secondary metabolites can be evaluated further.

2. Materials and methods

2.1. Culture condition

A. rhizogenes strain ATCC 15834 was used for hairy root induction. The bacterial strain was cultured on YEB (Yeast Extract Beef extract) broth supplemented with 5.0 mg/l rifampicin and incubated at 28 °C in dark for 48 h. Bacterial cultures were harvested at optical density of 0.6 and the pellet was collected by centrifugation at 6000 rpm for 10 min. The pellet was again re-suspended in 250 ml liquid MS medium, incubated at 28 °C in rotary shaker at 100 rpm for 4 h in dark and used for infection. *In vitro* culture of *P. thyrsiflorus* was developed in MS media supplemented with 5.0 mg/l BA and 50.0 mg/l adenine sulphate following protocol established by Kumari (2008). Aseptically grown shoot-tips, leaves and nodes were excised and used as explants for infection. The excised explants were wounded using sterile scalpel and co-cultured with bacteria in MS liquid media for 45, 75, 105, 135, 165, 195, 225 and 255 min. Explants were injured all over the surface by slits to facilitate the infection process before treatment with *A. rhizogenes*. Explants were then washed with 250 mg/l cefotaxime sodium salt supplemented MS medium followed by final rinse in sterile distilled water. Explants were then inoculated in solid MS basal media. The cultures were kept in the culture room maintained at 25 ± 2 °C temperature with 16 h light (at 3000 lx) and 8 h dark photoperiod regime.

2.2. Statistical analysis

Data were collected after 7, 14 and 21 d of inoculation to calculate transformation frequency as well as number of roots per explant. Data in 3 replicates were analyzed statistically using Completely Randomized Design (CRD) (Chandel, 1964). Standard Error of mean (SE_m) and Critical Differences (CD) were calculated.

For PCR analysis, plasmid DNA from *A. rhizogenes* strain (positive control), genomic DNA from transformed and non-transformed (negative control) roots of *P. thyrsiflorus* was isolated and used for PCR amplification. The isolation of plant genomic DNA was carried out using standard CTAB method (Doyle and Doyle, 1987). Isolated DNA was subjected to polymerase chain reaction (PCR) for the presence of *rol C* and *aux 1* genes in T-DNA region using the sequence specific primers (Table 1).

The PCR reactions were carried out in a total volume of 20 μl volume and consisted of 200 ng of DNA prepared from normal and hairy roots respectively as the template, 0.2 μM of each primer, 0.25 μM of dNTPs, 0.75 unit of Taq DNA polymerase (Sigma), 1× PCR buffer and 2.5 mM $MgCl_2$. Amplification cycle included initial denaturation for 5 min at 95 °C followed by 35 cycles of 3 s denaturation at 94 °C, annealing for 1 min at 46 °C, extension at 72 °C for 2 min and 12 min final extension at 72 °C in a thermocycler (Applied Biosystems 9700 thermocycler). The amplified PCR products were separated following gel electrophoresis on a 1.4% agarose gel stained with 0.5% (aqueous) ethidium bromide. The amplicons were compared with a DNA molecular weight marker 'PCR low ladder' (Sigma, USA). The gel was then visualized and photographed in a UV Transilluminator (Syngene Germany).

3. Results and discussion

3.1. Factors affecting transformation and hairy root formation

Wounded explants were cultured in liquid MS media containing *A. rhizogenes* (strain ATCC 15834) for 45, 75, 105, 135, 145, 195, 225 and 255 min. After 7 d of co-culture or infection, emergence of hairy roots was observed on all explants except node. Difference in mean percentage of root induction on shoot tip, node and leaf was observed in different co-culture periods. Effect of co-culture period as well as number of days in culture has been found significant for root induction percentage and number of roots per explants (Tables 2 and 3). Out of eight different co-culture periods, lowest induction percentage was observed after 45 min while it was highest after 225 min of co-culture. A gradual increase in induction percentage was observed in 75, 105, 135, 165 and 195 min of co-culture period. However, further co-culture up to 255 min showed decline in root induction percentage and number of roots per explants. Several authors reported that co-culture time has got significant effect on transformation of the explants. Xu et al. (1997) reported that co-culture time affects the transformation frequencies of alfalfa suspension cultures. Karmarkar et al. (2001) tested the ability of *in vitro* grown leaf segment, inter-node segment, shoot buds, seedling hypocotyls and callus of *Holostemma ada-kodien* to induce hairy roots. In *P. thyrsiflorus*, significant increase in percentage of root induction and number of roots per explants were found when the co-culture time was 225 min. At this co-culture period 60% of root induction was achieved after 7 d which was increased to 86.66% after 14 d and finally to 93.33% after 21 d (Table 2, Fig. 1a–f). However, no root induction was observed on control during these periods. Moreover, browning of explants started after 12 d.

Yonemitsu et al. (1990) and Trypsteen et al. (1991) reported that juvenility and nature of explants influence the *Agrobacterium* mediated transformation process. Giri et al. (2001) evaluated different strains of *A. rhizogenes* for induction of transformed hairy roots in *Artemisia annua* using shoot-tip meristem explants. Response of different explants as root, leaf, petiole, nodal parts of *Centella asiatica* and incubation time with *A. rhizogenes* 8196 strain was evaluated for hairy root induction and further growth (Gandi and Giri, 2012). Use of different explants of *Withania somnifera* (Saravanakumar et al., 2012) and *Gentiana cruciata* (Hayta et al., 2011) for hairy root induction revealed that that induction of root transformation was significantly dependent on the explants rather than the strains of bacteria used. In case of *P. thyrsiflorus*, transformation frequency was highest in shoot tip followed by node (86.66%) and leaf (73.33%) which is in accordance to the above findings. So far the number of roots per explants concerned, effect of