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Induction and Establishment of Hairy Root Culture of *Solanum xanthocarpum* using Agrobacterium rhizogenes

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Abstract

Hairy Root Culture is the new route for large scale secondary metabolite production because of their fast and plagiotropic growth, genetic and biochemical stability. In this report *Solanum xanthocarpum* was used to induce the hairy root cultures, for getting the benefits of the medicinal properties residing in the roots of the medicinally important plant. About 65.38% survivability was obtained for *S. xanthocarpum* axillary buds when cultured on MS medium supplemented with IBA (0.25mg/l), FAP (0.5mg/l) and 1% activated charcoal for explants preparation. As, a result of an effective infection of *Agrobacterium rhizogenes* carrying Ri plasmid with *rolC* gene, hairy root cultures were established, which were grown in culture media without hormones. The *Agrobacterium rhizogenes* MTCC532, strain used in the present study was capable of inducing hairy roots formation by simple stabbing method with high transformation frequency (75%) after 12 days of infection. Copious growth of Hairy Roots was observed in ½ MS liquid medium without hormones with growth index of 45 with doubling time of 7 days demonstrated a simple and effective method of establishment of hairy root cultures. PCR analysis showed amplification of the *rolC* gene in Hairy Roots, indicated the transformation event. Further, this study allows future scale-up of hairy roots in bioreactors for large scale production of secondary metabolites and increasing use of this as model system in metabolic engineering,

Keywords: Hairy Root Culture, *Solanum xanthocarpum, Agrobacterium rhizogenes,* Secondary Metabolites.

INTRODUCTION

Solanum xanthocarpum (Family: Solanaceae) is a perennial herb growing in dry plains and low hills characterized by the presence of steroidal alkaloids, solasodine and flavonoids. Solasodine (SD) has reported antiandrogenic activity, is the main constituent isolated from the berries and roots of the plant. SD serves as an important intermediate in synthesis of steroidal hormones and is a potential alternative to diosgenin, a precursor in the synthesis of steroidal hormones. Plant parts useful in treatment of hypercholesterolemia, prostatic carcinoma and breast cancer attributed to Carpesterol and β-Sitosterol[1-2]. Plant extracts exhibits excellent anti-asthmatic and antifungal activity.

Agrobacterium rhizogenes mediated hairy root production is a valuable tool for studies on the biosynthesis of secondary metabolites and for exploitation in metabolic engineering. Hairy roots are characterized by rapid growth and extensive branching in growth regulator-free medium. In general, they exhibit genetic stability and, in certain cases, they have the capability of synthesizing secondary metabolites normally present in roots and organs of the species of origin[3] . *Agrobacterium rhizogenes* inserts T-DNA from the Ri plasmid into the genomes of the host plants root cells, having the *rol* genes involved in root initiation and development[4-5]. For this reason, hairy roots have been induced in several medicinal and aromatic plants and cultured for the production of secondary compounds[6]. In a recent investigation by Pawar and Maheshwari extensive hairy roots were induced from leaf explants of *Solanum surratennes*[7] and then established on MS basal medium independent of exogenous supply of phytohormones. Wu and co-workers investigated induction and culture conditions

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for *Solanum nigrum* hairy roots from the cut edges of leaf explants and its solasodine production [8].

The aim of the present work was to initiate transformed root cultures of *Solanum xanthocarpum* by inoculating axenic explants with *A. rhizogenes* strain MTCC532 in a simple and effective way, and to establish cultures of transformed roots which could be used for the production of secondary compounds in future. The growth dynamics and molecular analysis by PCR, of the cultured transformed root were investigated.

MATERIAL AND METHODS

Explants Preparation

Plants were collected from Pharmacy Garden of Punjab University, Chandigarh, India. Axillary buds taken from young and healthy plants were washed thoroughly under running tap water and surface sterilized first with few drops of antiseptic solution in distilled water for 5-10 minutes to remove fungal contamination, and then treated with 0.1% (w/v) $HgCl_2$ for 8 min, followed by several rinses in sterilized distilled water aseptically. Then disinfected with 70% ethanol for 30 seconds, and finally a rinsed with sterilized distilled water. The surface sterilized explants were inoculated on Murashige and Skoog (MS) basal media[9] supplemented with 3% sucrose, 0.5 mg/L FAP, 0.25 mg/L IBA and 0.8% agar. After growth of explants in cultures, three subcultures at 20 days interval were made in the same media. The pH of all the media were adjusted to 5.6 before autoclaving at 121°C for 15 min. Cultures were incubated at 25 ± 2º C under fluorescent light with a 16 hour photoperiod. After 2 months, the in vitro grown shoots and leaves were used as explants for transformation.

Bacterial Culture Preparation

A. rhizogenes strain MTCC 532 (Microbial Type Culture Collection, IMTECH, Chandigarh), stored in sterile glycerol at −70°C, was cultured on YEB solid medium for activation of the strain and sub cultured 3 times on the same medium.

Induction and Establishment of Hairy Root Culture

The explants were infected by stabbing the bacteria with a sterilized toothpick. After stabbing explants were cultured in half strength MS solid medium supplemented with 3% sucrose in dark regime at 25º C for 48 hrs. Elimination of bacteria was then done by transferring the stabbed explants to ½ MS solid medium with antibiotic, ampicillin (0.5mg/ml) at 25º C in dark. Hairy Roots that developed, were transferred to antibiotic and hormone free ½ MS liquid medium at 25º C at 100 rpm on orbital shaker under dark and subcultured in fresh medium every 15 days. Inoculums of roots were transferred in aseptic conditions to 50 ml liquid MS medium, which corresponds to initial fresh weight (FW). The Growth Index was calculated by the ratio of final fresh weight to initial fresh weight. Doubling time was calculated by plotting a graph of $\log_2$ FW (g) v/s time (days) and then calculating the inverse of the slope for the linear part of the curve.

PCR Conformation Study

Plasmid DNA from A. rhizogenes strain and DNA from hairy roots was used for PCR amplification. Plasmid DNA was extracted using the alkaline lysis method and plant DNA was extracted following the method documented by Mariya-John[10]. Polymerase chain reaction was carried out using *rol C* gene specific primers. For this 50 ng plasmid DNA and DNA from non-transformed leaf tissues were taken as positive and negative controls, respectively. DNA from hairy roots was served as treatments. Each 25 µl reaction mixture contained 1X PCR buffer, 3.5 mM $MgCl_2$, 25 pmol of each forward (5'-ATGGCTGAAGACGACCTGTT-3') and reverse (5'-TTAGCCGATTGAAAACTT GCAC-3') primers with 0.2 mM dNTPs and 1 U of Taq DNA polymerase (Bangalore Genei Private Ltd. Bangalore, India). Amplification cycle included initial denaturation for 4 min at 94°C, followed by 30 cycles of 45 sec denaturation at 94°C, annealing for 1 min at 55°C, extension at 72°C for 2 min and 10 min final extension at 72°C in MyCycler™ (Bio-Rad). Amplified products were visualized under Gel documentation system after running the samples on 1.5% agarose gel.

RESULTS AND DISCUSSION

In the present study, axenic culture of *Solanum xanthocarpum* was established by *in vitro* propagation, using meristematic axillary buds. Nodal explants with meristematic axillary buds were cultured on MS medium supplemented with IBA (0.25 mg/l) + FAP (0.50 mg/l) and 1% activated charcoal. As *Solanum xanthocarpum* produces phenolic compounds which results in the browning of medium and darkening of the explants, 1% activated charcoal was supplemented with the medium which acts as antibrowning agent by absorbing the secreted phenolics by the explants (Figure1A).

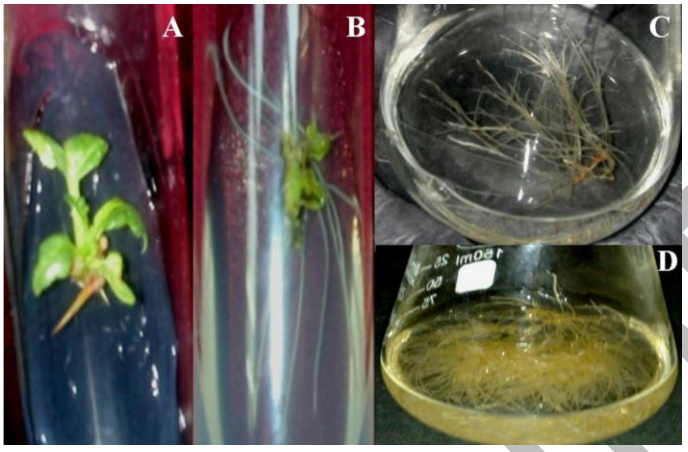

Figure 1: A) The axenic leaf explants of Solanum xanthocarpum, B) Roots emerged from the inoculation site of the leaf, C) Copious growth of Hairy Roots observed in liquid medium after 15 days, D) After 57 days.

After 20 days about 65.38 % survivability of plantlets and on average 1.3 shootlets/explants was obtained. Subculturing was done on the same medium routinely after 20 days. After subculturing light green healthy shootlets and small leaves without thorns were observed as compared to the adventitious shoots produced on MS medium fortified with BAP and kinetin[11]. After 30 days of subculturing, creamish white healthy roots (40%) were observed. In present work, no studies were carried out on rooting as only leaves were used as explants for the establishment of the Harry Root Culture, which was the sole objective.

For establishment of the HRC, the source of explants must be a true to type of the plant and should not have any genotypic variation. Therefore to maintain the genetic and biochemical stability in the HRs, axillary buds were preferred as explants for axenic cultures, to obtain clonal explants than the callus culture in which somaclonal variation are more common leading to plants with genotypic difference. Axenic cultures of *Solanum xanthocarpum,* was used to initiate transformed root cultures by inoculating axenic explants with *A. rhizogenes* strain MTCC532, and to establish cultures of transformed roots for the production of secondary compounds. About 75 % transformation frequency was observed after 12 days of infection by stabbing method which is very simple comparing to other reports. The bacterium was eliminated by culturing Hairy Roots developed at the site of infection on ½ MS solid medium with ampicillin (0.5 g/l) for 4 days (Figure1.B). Copious growth of HRs was observed in ½ MS liquid medium without hormones at 100 rpm on an orbital shaker, 15 days after the elimination of bacteria using ampicillin (Figure1.C). These transformed roots have several properties, such as fast and plagiotropic growth, a high degree of lateral branching. Inoculums of 0.2g roots were transferred in aseptic conditions to 50 ml liquid ½ MS medium, which corresponds to initial fresh weight (FW) (Figure1.D). After 57 days of culture the Growth Index was found to be about 45. Doubling time was calculated by plotting a graph of log2 FW (g) versus time (d) and calculating the inverse of the slope for the linear part of the curve. The doubling time was as observed from the plot was 7 days (Figure 2A). Wu and coworkers investigated induction and culture conditions for *Solanum nigrum* hairy roots and its solasodine production. The results showed that hairy roots could be initiated from the cut edges of leaf explants 5 days after inoculation with the strain of *A. rhizogenes* ATCC15834. PCR analysis of the transformed hairy roots showed amplification at 540 bp in hairy root samples and in positive control (plasmid), indicating the transformation

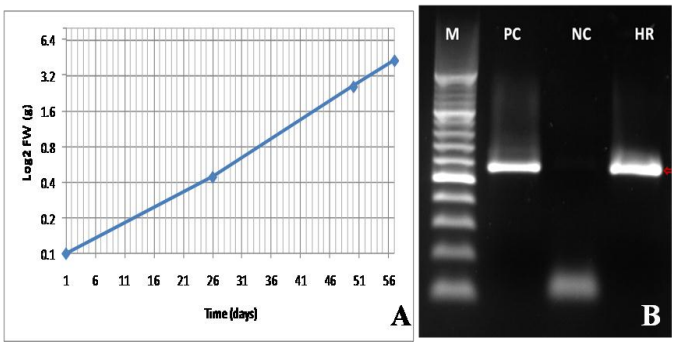

Figure 2 A) The graph of log_2FW(g) versus time (days) of the Hairy Roots cultured in liquid medium, B) PCR analysis of Hairy Roots: M, 100 bp DNA ladder; PC, Positive control; NC, Negative control; HR, Hairy Root samples.

event. But in negative control (non-transformed leaf tissues) there was no amplification (Figure2.B). This confirmed that hairy roots have developed because of the *rolC* gene integration. Mariya- John also confirmed transformation event through PCR amplification of *rol C* gene in hairy roots. Mariya-John reported that physiological factor, such as, hormone plays an important role in the formation of hairy roots and also indicated that source and concentration of carbon in the basal medium was one of the important factors for hairy root induction. But results of our studies suggest that these hairy roots were established on MS basal medium and their growth was observed to be independent of exogenous supply of phytohormones as also reported by Pawar and Maheshwari.

Most of the previous reports described coculturing to be one of the best methods for developing hairy roots. The *Agrobacterium rhizogenes* MTCC 532 used in the present study was capable of inducing hairy roots formation by stabbing with high transformation frequency (75%). This study demonstrated the transformation of *Solanum xanthocarpum,* leaves with *A. rhizogenes* and establishment of hairy root cultures. Moreover, Hairy Roots established in the present work will be the main source of metabolites extraction which are present in the roots of this plant like Solasodine, Carpesterol and β-Sitosterol and this could be exploited commercially. These results also demonstrated that the HRs produced could be used as source of enhanced production of secondary metabolites [12] and also for phytoremediation [13-14], in less time and at low cost. Scale up of Hairy Roots in bioreactors may prove to be a useful technique for large scale production of secondary metabolites and phytoremediation of industrial effluents and its increasing use as model system in metabolic engineering. However, further investigation is required to quantify the level of these metabolites in hairy roots as well as to enhance the production of hairy root biomass.

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