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AGROBACTERIUM RHIZOGENES MEDIATED INDUCTION OF HAIRY ROOTS IN COLEUS FORSKOHLII FOR EXTRACTION OF FORSKOLIN

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ABSTRACT

Coleus forskohlii, a medicinally important plant of the Labiatae (Lamiaceae) family was investigated for shoot organogenesis, direct regeneration and induction of hairy root using the soil bacterium MTCC 2364 strain of Agrobacterium rhizogenes. Nodal stem and leaf were initiated on basal MS medium with 6-benzyl amino purine (BAP) 0.5, 1.0, 2.0 mg/L. Shoot generated from such effective combinations was co-cultivated with the bacterium in vitro. Extensive hairy roots were induced from the shoots and leaf within 15-18 days. These roots were then established on MS basal broth medium. Maximum concentration of Forskolin on the specific day of culture of hairy roots was estimated and compared to the authentic standard Forskolin concentration in HPLC. The concentration of Forskolin was observed to be maximized in 14th day old culture (116 mg/Kg Dry Cell Weight) and the content was found to decrease gradually thereafter towards day 30.

KEYWORDS: Coleus Forskohlii, Hairy Root Culture, Agrobacterium Rhizogene, Forskolin.

INTRODUCTION

Coleus grayscale is a perennial herb of the Labiatae (Lamiaceae) family that includes the herbs, mints and lavenders. C. forskohlii, also known as Makandi, Mayini and Puravai, grows wild in warm, subtropical and temperate areas in India, Burma, Thailand, Bhutan and Nepal (Saha et al. 1980). It grows well at an elevation 600-1800 meters above sea level, in loamy and sandy loans soil with a pH of 6.4-7.9. The plant is valued for the production of Forskolin, a Labdane Diterpene compound which is used in the treatment of Glaucoma, Congestive Cardiomyopathy, Asthama (Valdes et al. 1987), Hypertension, Psoriasis, Eczema and obesity (Henderson et al. 2005). Forskolin is found in almost all parts of the plant, with roots being the main source (Shah et al., 1980). Forskolin acts by stimulating Adenylate cyclase, thereby increasing cellular concentrations of the second messenger cyclic AMP (cAMP) (Seamon et al. 1981). Forskolin content of natural of root (dry weight) varies from 0.04 – 0.44% (Viswakarma et al. 1988). Extraction of Forskolin from field crop is a difficult process and requires large area, equipments and man power. In vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants and practice are the key step towards commercial production. Hairy roots have several properties that have promoted their use of plant biotechnological applications. The soil-borne bacterium Agrobacterium rhizogenes is the causative agent of hairy root disease, in dicotyledonous plants. Infection with Agrobacterium rhizogenes displayed enhanced production of those secondary metabolites which occur naturally in untransformed roots, resulting in amounts of secondary compounds comparable or even higher than those present in intact roots (Sharp & Doran, 1990; Zárate, 1999). Agrobacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants (Tepfer, 1990), this infection is followed by the transfer of a

portion of DNA i.e. T-DNA, known as the root inducing plasmid (Ri-plasmid), to the plant cell chromosomal DNA. Inamdar et al. (1984) reported a comparison of thin-layer chromatography (TLC), gas– liquid chromatography (GLC), and high performance liquid chromatography (HPLC), for the quantitative estimation of Forskolin. Of the methods studied, normal- phase HPLC was determined to be the best procedure for the analysis of the extract of C. Forskohlii.

MATERIAL AND METHODS

Surface Sterilization and Culture

Young branches of Coleus forskohlii were collected from CIMAP Bangalore and were identified by Taxonomist. Explants were grown in pots with suitable soil conditions. Shoot tip explants was collected from potted plants and processed for aseptic culture. Surface sterilized by cleaning thoroughly under running tap water for 10 min, then detergent wash for 15 minutes and then rinsed 4-5 times tap water for 30 minutes. Wash with 70% alcohol for 3-5 second, followed by double distilled water wash for 3-4 times. Further, sterilization procedure was carried out inside laminar air flow chamber. The explants were then dipped into 0.1% bavistin for 45 minutes and finally treated with HgCl2 (0.05%) for 10 min, then add HgCl2 (0.1%) to this solution and wait for 5 minutes followed by washing with sterile distilled water for 4-5 times. After surface sterilization the explants were trimmed into small uniform pieces; and inoculated in MS supplemented media with hormones BAP (0.0, 0.5, 1.0, 2.0 mg/l), prepared before 48 hours of inoculation. All cultures were maintained at 20-25^{o}C in fluorescent tube light (40-80 πmol m^{-2} s^{-1}) for 7-10 days. These exploits were used for transformation using Agrobacterium rhizogenes.

Bacterial Strain

Wild type strain of A. rhizogenes (MTCC 2364) obtained from the National Bureau of Agriculturally Important Microorganisms (NAIMCC) coded with NAIMCC-B-00025, Stem gall, medium-179, 25^{o}C maintained in yeast extract mannitol agar (YEMA) medium containing (per liter) 1 g yeast extract, 10 g mannitol, 0.5 g K2HPO4, 0.2 g MgSO47H2O, 0.1 g NaCl, and 1.5% (w/v) agar, final pH 6.8$\pm$2 was used for transformation experiments)

Induction and Establishment of Hairy Root Culture

For transformation studies, A, the resurgence was incubated 25^{o}C for 36 hrs in a conical flask, on an arbitrary shaker at 200 RPM with yeast extract mannitol broth (YEMB) medium. The medium was then centrifuged at 3000 RPM for 5 min and the resultant cell suspension was resuspended in 5 ml of sterile MS medium. The leaf explants were trimmed and cut into small size (0.5 cm^2) and the stem were cut and wounded properly. Each explant was immersed in the bacterial suspension separately for 5 min, then-blotted dry on sterile filter-paper to remove excess bacteria and placed back in their original culture plates. After 2 days of co-culture at 26°C in the dark, the explants were transferred onto hormone-free media containing 500 mg/l Cefotaxime to eliminate bacteria and then incubated at 25±2°C under a 16 h light and 8 h dark photoperiod. Extensive hairy roots were induced from the shoots and leaf within 15-18 days. These roots were then established on MS basal broth medium.

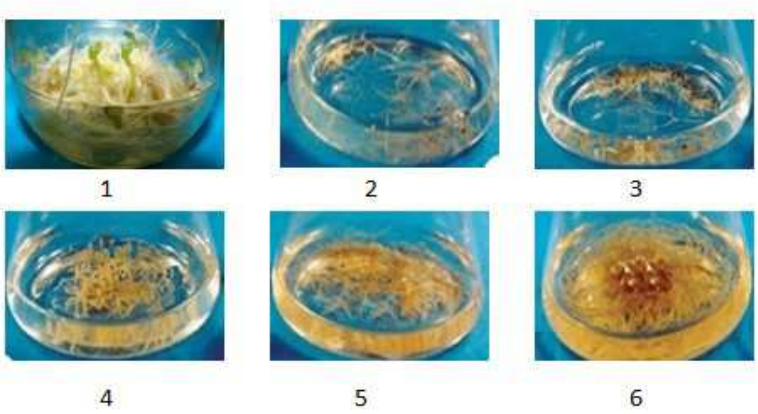

Figure 1

Hairy root induction by a cut end method using A. Resurgence in Coleus forskohlii 2: Copious growth of the Hairy root cultures of the developed MS liquid medium without hormones at 100 RPM on orbital shaker in picture 2,3,4, and 5 for day 5,10, 15, 25 and 45 respectively

Extraction and HPLC Analysis

Hairy roots were sampled in an alternate day from day 0 to day 30 after hairy root induction. Forskolin extraction from hairy roots was carried following protocol as described by Hamerslag (1950) and Walaszek et al. (1952). Twenty grams of hairy roots were washed thoroughly with sterile distilled water, blotted dry followed by freeze drying and then extracted using ethyl acetate at 70º C for 2 hours. The ethyl acetate extract was filtered and concentrated in vacuum. Quantitative determination of the alkaloid was carried out by HPLC. U-boat 25mg of standard Forskolin was weighed accurately into a 25ml volumetric flask, dissolved and diluted to volume with methanol and mixed well. One gram of sample was weighed into a 100ml flask; 50ml of methanol was added and refluxed for 30 minutes. The process was repeated with 3x 50ml of methanol. All the methanol extracts were combined and diluted to 250ml with methanol, mixed and filtered through Whatman No. 42, to get a clear solution. Mobile phase was prepared by mixing Hexane, Ethyl acetate and Methylene chloride in the proportion of 70:20:10 and deduced. The liquid chromatography (Shimadzu Corporation, Kyoto, Japan) was equipped with a 210 nm UV detector and C_{18} column (250 x 4.6mm: M/s Spinco Biotech Pvt. Ltd., Bangalore, India). The mobile phase was pumped at the rate of 0.8 ml/min with a back pressure of 200 psi. The injector and the detector were flushed with the mobile phase. The refractive index of the detector was set at 4X and the potentiometer chart speed was set at 0.5 cm/min. The column was equilibrated for half an hour. The flow rate was about 1.0 ml per minute. The standard preparation was chromatographed and the peak response for Forskolin was recorded. Equal volumes of (10µl) of the standard preparation and sample preparation were injected separately. Recorded the chromatograms and measured the responses for the peak corresponding to Forskolin. The statically analysis was carried out using ANOVA (Analysis of variance technique) in the present study.

RESULT AND DISCUSSIONS

The hairy root cultures were successfully induced from leaf plant within 10 days after incubation with MS media. After 2-3 weeks numerous hairy roots appeared at the inoculation site. The obtained hairy roots were maintained in media containing antibiotic cefataxim (500mg/L) the elimination of bacteria strain was successful after three subcultures done at

3 week intervals. Then the rats were placed in antibiotic free media. The hair roots were white in color and highly branched. Rapid growth started after 10 days continued until 30 days. The hair root was sampled alternate days and sent to HPLC estimation. The maximum production of forskolin 0.0116% (116mg/kg DCW) from hairy root culture was obtained on the 14th day. (Graph-1) p-value was calculated <0.005. However, the growth of hairs root culture was sustained at the same rate, but the content of forskolin was declined after 16th day. However, up to 25 days of incubation are ideal for the production of forskolin in hairy root culture. Forskolin production in vitro cultures of C. forskohlii has been reported by Mersinger et al. (1988), Krombholz et al. (1992) and Sen et al. (1992), and the maximum forskolin productivity was 0.073%, 0.1% and 0.01%, respectively. In this present study, identification & quantification of Forskolin in A. rhizogene (MTCC 2364) transformed hairy roots of Coleus forskohlii was done by HPLC. The amount of forskolin in hairy root of Coleus forskohlii determined here is promising.

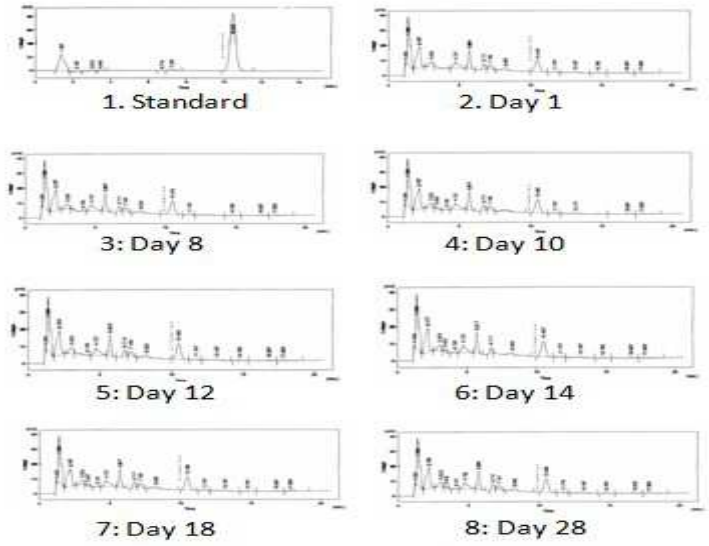

Figure 2

Figure 1: Liquid chromatogram of forskolin obtained (1) in the standard stock solution and (2, 3, 4, 5, 6, 7, 8) in a hairy root culture of coleus forskohlii and contain forskolin in day 1,8,10,12,14,18, and 28th day.

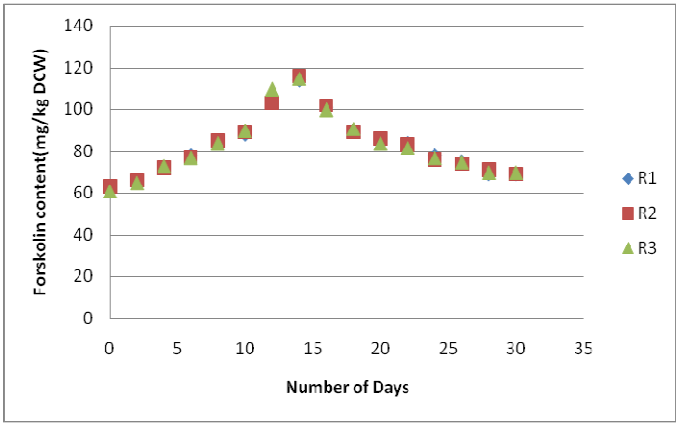

Figure 3

Figure 2: Forskolin production in hairy root cultures of Coleus forskohlii (Forskolin content (mg/kg DCW)

CONCLUSIONS

In this study, we have demonstrated an efficient Agrobacterium rhizogenes mediated transformation of Coleus forshkolin. Using this standardized protocol, hairy roots can be harvested in large scale, in turn, large quantities of important alkaloids can be produced in future investigations. Further, use of electors and precursors in the production of forskolin from hairy roots culture can be studied which may increase the content of secondary metabolite as per reported in Dhatura strannnium (Ballica R et al, 1993). Further, there is an ever growing demand of herbal medicines, so scale up of Hairy roots of the Coleus forskohlii in practice will be a useful technique for large scale production of secondary metabolites in less time and at low cost.

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