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AGROBACTERIUM RHIZOGENES MEDIATED HAIRY ROOT CULTURE AND GENETIC TRANSFORMATION OF AN ENDANGERED MEDICINAL PLANT OF *DECALEPIS HAMILTONII* WIGHT & ARN.

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ABSTRACT

The total dry matter content of 2-hydroxy-4-methoxy benzaldehyde production by hairy root culture of *Decalepis hamiltonii* from *in vitro* internode and roots explants inoculated with *Agrobacterium rhizogenes*. The addition of acetosyringone containing basal Murashige and Skoog's (MS) liquid medium fortified with α-naphthaleneacetic acid (NAA) and indole 3-butyric acid (IBA) are improved hairy root production. PCR analysis of Ri plasmid on transformed internode and root tissues showed DNA amplification at 780 bp amplified in internode, root derived hairy roots. The highest biomass of dry matter content (25.36 and 24.12 g) was observed on liquid MS medium containing 0.3 mg/L NAA, co-cultivated with *A. rhizogenes* on the internode and root explants. After that the internode and root explants were inoculated on MS medium supplemented with 0.3 mg/L IBA was improved biomass content of dry matter 23.56 and 22.91 g respectively. The dry matter content of biomass was extracted with 1 mg/ml methanol to measure the total content accumulation of 2-hydroxy-4-methoxy benzaldehyde in hairy roots cultures confirmed by using HPLC.

Keywords: *Decalepis hamiltonii*, *Agrobacterium rhizogenes*, hairy root, genetic transformation

INTRODUCTION

Decalepis hamiltonii (*Asclepiadaceae*) is a monogeneric climbing shrub endemic to the Deccan peninsula, which is used as a culinary spice due to its highly aromatic roots. It is useful as an appetite, blood purifier, preservative and as a source of bio insecticide for stored food grains **(George *et al*., 1999a; George *et al*., 1999b)**. These people procure root and habitually carry them and chew the same whenever the digestion may seek relief. Besides treating indigestion the roots have been used locally to stimulate the appetite and to relieve flatulence and act as a general tonic and also consumed as food which helps in maintaining their health status **(Vedavathy, 2004; Samydurai *et al*., 2012)**. In chemical constituents, tuberous roots of the plant are closely similar to that of *Hemidesmus indicus* and *Decalepis hamiltonii* an allied species which are widely used in the traditional system of medicine as a blood purifier and as a flavouring agent for the preparation of soft drinks and bakery products **(George et al., 1999)**. Recently many authors reported that this plant has been potential for bioactive chemical constituents and biological activity of antibacterial, antioxidant, anti-inflammatory **(Mohana *et al*., 2009; Thangavel *et al*., 2011; Ashim Kumar *et al*., 2011; Samydurai *et al*., 2012)**.

Advances in plant cell tissue culture, combined with improvement in genetic engineering, specifically transformation technology has opened new avenues for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances **(Hansen and Wright, 1999)**. Transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production from the wild and commercial medicinal plants. They are unique in their genetic and biosynthetic stability, faster in growth and more easily produced wide range of bioactive chemical compounds has been synthesized **(Giri and Narasu, 2000; Ahlawat *et al*., 2012)**. In order to obtain high yields suitable for commercial exploitation, efforts have focused on isolating the biosynthetic activities of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains, and employing precursor feeding, transformation methods.

MATERIAL AND METHODS

Bacterial strains and culture

Agrobacterium rhizogenes MTCC 532 (Microbial type culture collection, IMTECH, Chandigarh) was inoculated on Luria-Bertani (HiMedia, Mumbai, India) agar medium kept in incubation room at 25°C for 24 h. Then the grown bacterial inoculum was again subcultured on LB broth suspension kept in orbital shaker at incubation room. The bacterial suspension was transferred to a sterile centrifuge tube and centrifuged at 5000 rpm for 15 minutes. The biomass of bacteria was suspended in liquid MS media supplemented with 3% sucrose (The optical density of bacterial suspension ranges was 0.4-0.6 at 600 nm). This suspension was used for the transformation of *in vitro* derived internode and root explants. In this study, the effect of different growth regulators and acetosyringone compounds on hairy root growth and production of 2-hydroxy-4-methoxy benzaldehyde from *Agrobacterium* mediated hairy root cultures of *D. hamiltonii*.

Establishment of hairy roots

The induction of hairy roots was culture on 100 ml liquid MS medium supplemented with different concentrations of NAA and IBA (0.1 to 0.4 mg/L) containing 3% sucrose and addition of 200 mg/L acetosyringone and cefotaxime 250 mg/L. The pH was adjusted prior to 5.6 to 5.8, autoclaved at 121°C for 20 minutes. Twenty days old *in vitro* grown plants of *Decalepis hamiltonii* were used as the explant of internode and root were inoculated with *A. rhizogenes*. Hairy root cultures were maintained at 25°C on a gyratory shaker at 80 rpm in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 µmol s^{-1} m^{-2} and 16 h photoperiod. After 3 weeks of culture, hairy roots were harvested and the dry weight was determined. Each experiment was carried out with 3 flasks per culture condition and repeated twice.

PCR analysis of putative transgenic plants

Plasmid DNA from the *Agrobacterium rhizogenes* and transformed stem and root induced hairy roots were used for PCR amplification. Plasmid DNA was extracted using Geneelute™ HP plasmid miniprep kit (Sigma Chemicals Company, USA) and plant DNA was extracted following method documented by **Mariya John (2007)**. Polymerase chain reaction (PCR) was carried out using *rol* C gene specific primers following the procedure given elsewhere **(Bulgakov *et al*., 2002)**. For this 50 ng plasmid DNA and DNA from non-transformed roots were taken as positive and negative controls, respectively. DNA from transformed internode and root derived hairy roots served as treatments.

Determination of Dry matter content

In order to determine biomass weight, hairy roots were harvested and rinsed with distilled water, then blotted to remove any excess water and weighed. Dry weight was measured gravimetrically after drying the roots for 48 h at 28°C room temperature.

$$\text{Dry matter content \%} = \frac{\text{Dry weight of the hairy roots}}{\text{Fresh weight of the hairy roots}} \times 100$$

HPLC analysis of hairy roots

Extraction of secondary metabolites from *in vitro* cultured hairy roots was done by the method of **Sangwan *et al*. (2005)**. Roots were harvested, washed with distilled water and extracted with 75% aqueous methanol (x 3 10 mL) and filtered. The filtrate was defatted with an equal volume of n-hexane (x 3). The defatted extract was partitioned with equal volumes of chloroform (x 3). The chloroform fractions were pooled, evaporated to dryness, dissolved in 5 mL methanol (HPLC grade), clarified using Millipore filters (0.22µm) and subjected to HPLC analysis.

Data analysis

Triplicates were maintained in all the treatments and they were repeated twice. All the data were analyzed statistically. Hairy root induction on *in vitro* internodes and roots using different concentrations of plant growth regulators (PGRs) were analyzed by Duncan multiple range test (DMRT). The effects of different PGRs on hairy root formation were compared with controls and the data were subjected to one way analysis of variance (ANOVA).

RESULTS

Effect of PGRs on hairy root production

In the present study, the MS liquid medium containing different concentration of plant growth regulators were incorporated with *Agrobacterium rhizogenes* influence on hairy root formation and genetic transformation was observed. Among the different concentrations of plant growth regulators, naphthalene acetic acid and indole butyric acid was significantly enhanced genetically transformed hairy roots at lowest concentration, which also declined hairy root production at higher concentrations. *Agrobacterium rhizogenes* co-cultivated with internode segments produced higher percentage of hairy root (96.9) was observed on liquid MS medium containing 0.3 mg/L NAA and 95.7% of hairy root formation was obtained on liquid MS medium fortified with 0.3 mg/L indole butyric acid. The hairy root induction was absent on liquid MS medium without strains of *A. rhizogenes* and addition of plant growth regulators. Therefore, naphthalene acetic acid and indole butyric acid containing MS liquid media was found to be ideal for the rapid production of hairy root production.

PCR analysis of transformed hairy roots

The presence of Ri plasmid gene in the induced hairy root lines was detected by PCR analysis. *Agrobacterium* mediated hairy root transformants indicative 780 bp Ri plasmid gene product amplified. PCR analysis of Ri plasmid transformed root tissues showed amplification at 780 bp in internode (Lane 3), root derived hairy root samples (Lane 4) and in positive control of Ri plasmid containing *Agrobacterium rhizogenes* (Lane 1). But in negative control (non-transformed hairy root) amplification was not recorded (Lane 2).

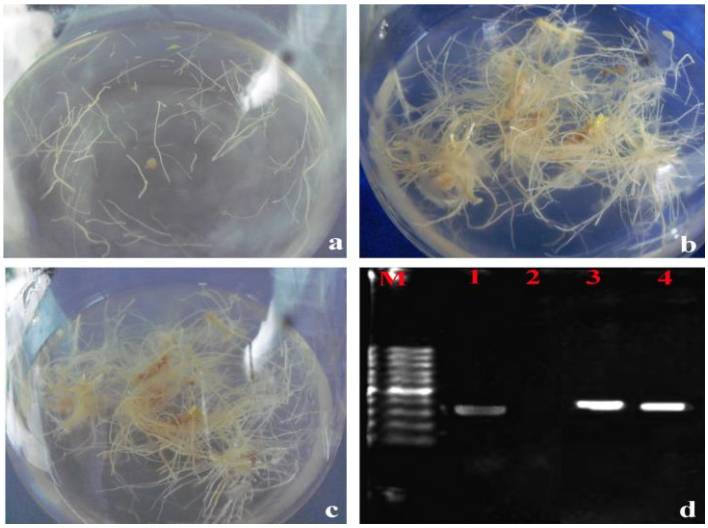

Figure 1 Showing the hairy root induction and DNA amplification of *Agrobacterium* mediated transformed hairy roots; a- control; b-internode; c-root explants and d- DNA amplification

Dry matter content of *Agrobacterium* mediated hairy roots

The first week of hairy root culture period on liquid media, a brown material accumulated around the Erlenmeyer conical flask and the medium turned to light golden brown. The hairy roots were transferred to fresh media at the end of every week. After three weeks *Agrobacterium rhizogenes* induced with hairy roots were taken out and washed with sterilized distilled water. The highest percentage of dry matter content (25.36 and 23.56) was observed in 0.3 mg/L naphthalene acetic acid and 0.3 mg/L indole butyric acid produced significant amount of dry matter content from internode segments and root explants also produced noticeable amount of dry matter content of 24.12 and 22.91% were recorded in 0.3 mg/L NAA and IBA (Figure 2 & 3).

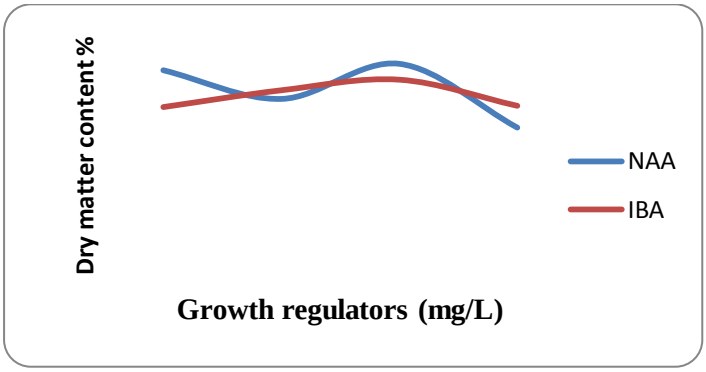

Figure 2 Percentage of dry matter content of 2-hydroxy-4-methoxybenzaldehyde on hairy root cultures of *Decalepis hamiltonii* from *in vitro* internode explants
Legend : NAA- α-Naphthalene acetic acid, IBA-Indole 3-butyric acid

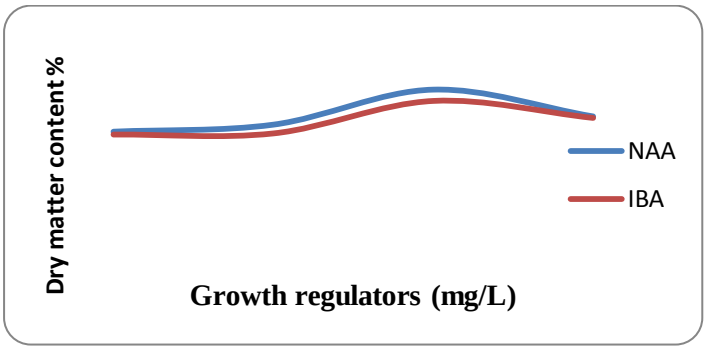

Figure 3 Percentage of dry matter content of 2-hydroxy-4-methoxybenzaldehyde on hairy root cultures of *Decalepis hamiltonii* from *in vitro* root explants
Legend: NAA- **α**-Naphthalene acetic acid, IBA-Indole butyric acid

Quantification of 2-hydroxy-4-methoxybenzaldehyde

The content of 2-hydroxy-4-methoxybenzaldehyde was quantified in *Agrobacterium rhizogenes* mediated hairy roots of *Decalepis hamiltonii*. The result of analysis for aromatic compounds by HPLC was shown in figure 4, and also HPLC resolved the samples in main peak area of 44.6%. These highest peaks were found to be 2-hydroxy-4-methoxy benzaldehyde produced in hairy root cultures from *Decalepis hamiltonii*.

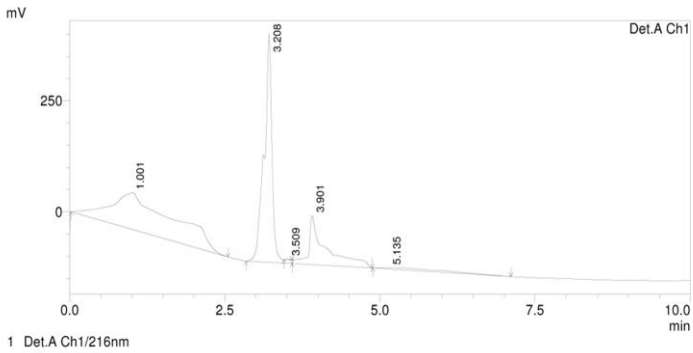

Figure 4 The HPLC analysis of 2-hydroxy-4-methoxybenzaldehyde from hairy root production of *Decalepis hamiltonii*
The highest peak area (3.208) is 2-hydroxy-4-methoxybenzaldehyde

DISCUSSION

The main objective of this investigation was to establish hairy root culture in an important woody climber plant *Decalepis hamiltonii* using *Agrobacterium rhizogenes*. In the present study, the highest percentage (96.9) of hairy roots was recorded in internode explant followed by root explant (93.3%). The hairy root cultures established in liquid MS medium fortified with growth regulators at low concentration, induced maximum production of yield. The present study provides a more effective approach for commercially large-scale production of volatile oil compounds of 2-hydroxy-4-methoxybenzaldehyde by cultivating *D. hamiltonii* plants in wide fields instead of using the hairy root systems as bioreactors. To the best of our knowledge and based on literature survey and citation it is assured that this is the first report on the hairy root culture of *Decalepis hamiltonii* from its internode and root explants.

Hairy root cultures are more efficient than normal root cultures, due to their genetic and biochemical stability for long period and are ideal for introducing genes to elevate the development of secondary metabolites. However, normal root culture system is an alternative method for those species, which are recalcitrant to *Agrobacterium rhizogenes* infection. **Thimmaraju *et al*. (2008)** reported an increase in the auxin content in the transformed tissues of *Beta vulgaris*. *Solanum dulcamara* plants hairy induced by Ri plasmid of *Agrobacterium rhizogenes* exhibited high level of yield production in auxin and cytokinin treatment as compared with non transformed roots **(Hashem, 2009)**. Acetosyringone are defined as derivatives of amino acids, which served as a nutrient source for the invading bacterium and enhance the transformation rate **(Joseph Lopez et al., 2004)**. Similarly, the 9 fold increased hairy root production of *Brugmansia candida* induced by acetosyringone elicitors of accumulation of catechin in hairy roots **(Mariya John, 2009)**.

In Genetic engineering modified the tropane alkaloid biosynthetic pathway via over expressing both *pmt* and *h6h* in *A. belladonna* hairy roots demonstrated that the metabolic engineering strategy of breaking can facilitate accumulation of tropane alkaloids in *A. belladonna* **(Yang *et al*., 2011; Liu *et al*., 2010)**. *Eurycoma longifolia* induced hairy roots were successfully initiated, through *Agrobacterium rhizogenes* infecting the hypocotyls region of this plant is highly beneficial mainly to the pharmaceutical industry as the hairy root induction increased the amount of valuable secondary metabolites **(Monica Danial *et al*., 2012)** and anthraquinone production was found to be different from that in the hairy roots of *Rubia species* **(Ercan, 1999; Park *et al*., 2009)**. The polymerase chain reaction performed after that the amplified genomic DNAs of Ri plasmid, transformed, non-transformed hairy roots and control. **Liu *et al.* (2010)**, reported in *Atropa belladonna* and integration of the *pmt* and *h6h* genes into the genomic DNA of transgenic hairy roots were confirmed by genomic polymerase chain reaction were observed at 461 and 626 bp respectively. The integration of the TL-DNA (*rol* gene) region of the pRi plasmid was confirmed by polymerase chain reaction analysis of the gene located in hairy root tissues of *Artemisia annua* **(Ahlawat *et al*., 2012)**.

Finally, the *Agrobacterium* mediated hairy roots were analyzed by high performance liquid chromatography (HPLC) confirmed in volatile oil compound of 2-hydroxy-4-methoxybenzaldehyde indicated highest peak area at 44.6%. Whereas, Thangadurai *et al*. (2002) estimated that the wild root extracts of *D. hamiltonii* contained only 37.45%, 2-hydroxy-4-methoxybenzaldehyde. Hence, it is proved that *A. rhizogenes* mediated hairy root induction could improve the secondary metabolite, 2-hydroxy-4-methoxybenzaldehyde. Similarly **Rahnama *et al.* (2008)** reported that *Agrobacterium rhizogenes* mediated hairy roots of *Silybum marianum* was highest biomass content of silymarin production were determined by high-performance liquid chromatography. Hairy root cultures leading to the production of a bioactive compound, plumbagin and it was confirmed by using thin layer chromatography **(Yogananth and Jothi Basu, 2009; Gangopadhyay *et al*., 2010)**.

CONCLUSION

This study emphasizes on the development of *Agrobacterium* mediated hairy root cultures of *Decalepis hamiltonii* with special attention to increase the volatile compound, 2-hydroxy-4-methoxybenzaldehyde. Genetic confirmation of transformed culture indicates that the hairy root cultures have partial integration of Ri-T DNA. The influence of different plant growth regulators on hairy root growth also increases the amount of bioactive compound. In order to develop a commercial production of aromatic volatile compound, 2-hydroxy-4-methoxybenzaldehyde was helpful to pharmaceutical and food industry in various aspects.

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