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Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation of spine gourd (*Momordica dioica* Roxb. ex. willd) for the assessment of phenolic compounds and biological activities

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ABSTRACT

An efficient protocol for hairy root induction of spine gourd (*Momordica dioica*) was established using *Agrobacterium rhizogenes* (KCTC 2703). This study evaluates the phenolic compound production, antioxidant and antimicrobial (antibacterial, antifungal and antiviral) activities of transgenic hairy root cultures in *M. dioica* . Hairy roots were induced from leaves, petiole, and internodal explants. Molecular analysis of PCR and gene sequencing using specific primers of *rolC* and *aux1* revealed T-DNA integration in the hairy root clones and RT-PCR analysis confirmed the expression of hairy root inducible genes (*rolC* and *aux1*). The greatest biomass accumulation of hairy roots on MS liquid medium supplemented with 3% sucrose was observed at 22 days. Ultra-HPLC was used to compare the individual phenolic compound contents of transgenic and non-transgenic roots. Moreover, transgenic hairy roots efficiently produced several phenolic compounds, such as flavonols, hydroxycinnamic acid and hydroxybenzoic acid derivatives. The total phenolic, flavonoid contents and biological (antioxidant, antibacterial, antifungal and antiviral) activities were higher in hairy roots compared to non-transformed roots. These results demonstrate the greater potentiality of *M. dioica* hairy root cultures for the production of valuable phenolic compounds and for studies of their biological activity.

1. Introduction

Momordica dioica Roxb. ex. Willd (Family: Cucurbitaceae) is a high nutritional value of wild edible vegetable and also highly used for the traditional medicine (Bharathi et al., 2014). It is commonly known as spine gourd or small bitter gourd or teasle gourd is an annual or perennial dioecious climber. It has native of tropical regions on Africa, South America and Asia with extensive distribution in Bangladesh, China, India, Japan, and Pakistan (Thiruvengadam and Chung, 2011; Talukdar and Hossain, 2014). Spine gourd contains significant amount of alkaloids, steroids, triterpenoids, glycosides, saponins, carotenoids, polyphenols, vitamins, and other health promoting phytochemicals, these may be helpful used for antioxidant, diabetes, cancer, neurodegenerative diseases, asthma, leprosy, hepatoprotective, analgesic, postcoital anti-fertility, nematocidal, jaundice, bleeding piles, anti-allergic, anti-malarial, anti-feedant, anti-bacterial, anti-fungal and anti-viral activities (Thiruvengadam et al., 2013; Talukdar and Hossain, 2014). Seed dormancy and pre-flowering sex determination are major controlling factor of commercial cultivation in spine gourd (Bharathi et al., 2014). The production of bioactive compounds through *in vitro* culture has been important and promising aspect of modern biotechnology. To meet the increasing demand for plants utilized in the nutraceutical, pharmaceutical, and cosmetical industry, much of the recent research has focused on the development of *in vitro* tissue or hairy root culture techniques as a useful alternative to improve the yield of bioactive metabolites in spine gourd.

Hairy root cultures induced through *Agrobacterium rhizogenes*-mediated transformations have developed as potential biotechnological system because these cultures fast growth rates, ease of maintenance, genetic stability, large scale biomass production without the need for external application of phytohormones and ability to synthesize a vast array of valuable secondary metabolites (Srivastava and Srivastava, 2007; Chandra and Chandra, 2011). Till date, hairy root cultures have been studied for the production of secondary metabolites for used as pharmaceuticals, nutraceuticals, food additives and cosmetics (Srivastava and Srivastava, 2007; Chandra and Chandra, 2011). Previously, it was reported that

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hairy roots enhanced the amount of saponin in *Bacopa monnieri* (Majumdar et al., 2011), anthroquinones in *Polygonum multiflorum* (Thiruvengadam et al., 2014a), total phenolic content in *Solanum lycopersicum* (Singh et al., 2014), polyphenols in *Momordica charantia* (Thiruvengadam et al., 2014b), and glucosinolates in *Arabidopsis thaliana* (Kastell et al., 2015). However, there are no reports on the induction of hairy roots, and production of phenolic compounds and also their biological activities from hairy root cultures of spine gourd. The main goal of the present investigation was to develop a biotechnological system for producing bioactive phenolic compounds of *M. dioica* as an alternative to harvesting the wild plant. First time, we have successfully established an efficient protocol for hairy root cultures of *M. dioica* for the production of individual phenolic compound profiles (flavonols, hydroxycinnamic and hydroxybenzoic acids) using by UHPLC analysis. In addition, we optimized the different liquid nutrient media and various concentrations of sucrose on hairy root growth, in terms of fresh and dry biomass accumulation in *M. dioica* . Finally, we evaluated the total phenolic, flavonoid contents, antioxidant and antimicrobial (antibacterial, antifungal and antiviral) activities from transgenic hairy roots and non-transgenic roots of spine gourd.

2. Material and methods

2.1. Collection plant materials

Tubers of *M. dioica* Roxb. ex. Willd (one-year-old) were collected from the Semmalai hills, Western Ghats (altitude 300–600 m) and developed in the botanical garden at Kulathur, Tamil Nadu, India (Thiruvengadam et al., 2013). The explants of leaves, petiole, and internodals were washed with a detergent solution for 5 min and rinsed with running tap water for five times. After being soaked in 70% (v/v) ethanol for 1 min, then explants were rinsed in distilled water, further, sterilized with 1.0% (v/v) sodium hypochlorite solution for 10 min, and rinsed repeatedly with sterilized distilled water. The explants were cut into small pieces of leaves (~10-mm^2), petiole, and internodal (~0.5-cm long) in size.

2.2. Hairy root induction by Agrobacterium rhizogenes and proliferation of hairy root cultures

Leaves, petiole, and internodal explants were infected with bacterial culture (OD600 nm = 1.0) of *A. rhizogenes* strain KCTC 2703 (Sivakumar et al., 2005) for 30 min. Thereafter, they were blotted dry on sterilized tissue paper, co-cultured on MS solidified medium and incubated under dark conditions at 25 ± 2 °C for three days. The co-cultured explants were then washed thoroughly with sterilized distilled water and transferred to a MS solid medium supplemented with 300 mg/L cefotaxime (Duchefa Biochemie, Netherlands). Root cultures were incubated under 16 h light/8 h dark provided by 40 W white fluorescent tubes (40 μmol m^{-2} s^{-1}) at 25 ± 2 °C for 25 days. The aseptically excised roots (2–3 cm long) were subcultured individually into MS liquid medium, supplemented with 3% sucrose and 300 mg/L cefotaxime. The cultures were kept on an orbital shaker (100 rpm) and incubated under the same conditions and subcultures of roots were done by every 12 days. The cefotaxime level was gradually reduced to 200 and 100 mg/L during the second and third subculture, respectively. After third subculture, roots were transferred on MS liquid medium without cefotaxime. Non-transformed roots were excised from *in vivo* grown plants cultured on MS liquid medium.

2.3. Optimization of growth index in the hairy root cultures

Single hairy roots (300 mg fresh mass) were excised and cultured in MS liquid medium supplemented with 3% sucrose. Growth kinetics at different time intervals (7, 15, 22, and 30 days) was examined to optimize biomass accumulation. Full and half strength of MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), NN (Nitsch and Nitsch, 1969), and LS (Linsmaier and Skoog, 1965) media and different concentrations of sucrose (1, 2, 3 and 4%) were tested to find a combination that resulted in the highest root biomass. The cultures were kept under continuous agitation at 100 rpm in an orbital shaker and incubated at 25 ± 2 °C with a 16 h light/8 h dark (40 μmol m^{-2} s^{-1}) supplied by 40 W white fluorescent lamps. The biomass of hairy roots was assessed at 22 days of culture. The roots were separated from the media and their fresh mass (FM) was determined later they were washed with distilled water and the excess surface water blotted away. Dry mass (DM) was noted after the roots were dried at 60 °C until a consistent weight was observed. The growth ratio was determined as GR = growth ratio is the quotients of the dry mass of harvested biomass and the dry mass of the inoculum.

2.4. Molecular characterization of hairy roots

2.4.1. Polymerase chain reaction (PCR)

Genomic DNA extracted from transgenic root clones and non-transgenic roots of *M. dioica* were used the DNA isolation kit (Fermentas Life Sciences, USA). The amplification reaction was carried out in GeneAmp PCR system DNA thermal cycler (PerkinElmer, USA) using 22-mer oligonucleotides as primers. A primer pair of 5′-ATGGCTGAAGACGACCTGTGTT-3′ and 5′-TTAGCCGATTGCAAACTTGCAC-3′ was used to amplify a ~500-bp fragment of the *rolC* gene (Sivakumar et al., 2005) and 5′-CCAAGCTTGTCAGAAAACTTCAGGG- 3′ and 5′-CCGGATCCAATACCCAGCGCTTT-3′ was designed to amplify a ~815-bp fragment of the *aux1* gene (Medina-Bolivar et al., 2007). In addition, primers (5′-ATGCCCGATCGAGCTCAAGT-3′ and 5′-CCTGACCCAAACATCTCGGCT-3′), amplifying a fragment of ~338 bp were used for detecting the *virD2* gene (Medina-Bolivar et al., 2007). The reaction mixture consisted of 1 μL of 1 unit *Taq* polymerase, 2.5 μL of 100 nM dNTP, 1 μL of 20 pM primer, 1 μL of 20 ng template DNA and 2.5 μL of 10X reaction buffer plus sterile distilled H_2O for a final volume of 25 μL. PCR was performed under the following conditions for *rolC*, initial denaturation at 94 °C for 4 min, followed by 30 cycles of amplification (94 °C 1 min, 60 °C 1 min and 72 °C 2 min) and 5 min at 72 °C; for *aux1*, initial denaturation at 95 °C for 3 min, 30 cycles of amplification (95 °C 30s, 58 ° C 30 s and 72 °C 1 min) and 72 °C for 10 min; for *virD2*, initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C 30 s, 56 °C 30 s and 72 °C 45 s) and 10 min at 72 °C. PCR results were checked using agarose gel electrophoresis with *Hind* III-digested λDNAmaker, detected by ethidium bromide staining, and photographed using the gel documentation system (Bio-Rad, USA).

2.4.2. Gene sequencing

The amplified PCR product (~500 bp) was extracted using the MinElute Gel Extraction kit (Qiagen, Germany) following the manufacturer's instructions. The eluted product was commercially sequenced (Macrogen, Korea) from both the orientations of gene to confirm the presence of the *rolC* gene in transformed hairy roots.

2.4.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using an RNA isolation kit (Fermentas Life Sciences, USA) from transgenic root clones and non-transgenic roots. RT-PCR was carried out with a Revert-Aid™ first strand complementary DNA (cDNA) synthesis kit (Fermentas Life Sciences, USA) following the manufacturer's instructions. The same