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Development of transgenic hairy roots and augmentation of secondary metabolites by precursor feeding in *Sphagneticola calendulacea* (L.) Pruski

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ABSTRACT

Sphagneticola calendulacea (L.) Pruski [synonym *Wedelia chinensis* (Osbeck) Merrill] is an important medicinal plant that possesses a variety of bioactive secondary metabolites. A stable transgenic hairy root culture has been developed using engineered *Agrobacterium rhizogenes* strain LBA1334 harboring pCAM:2 × 35S:gusA binary vector, to achieve high yield of secondary metabolites. Putative transgenic hairy roots appeared with highest transformation frequency (66.67%) when leaf explants were immersed in *A. rhizogenes* suspension for 10 min and co-cultivated for three days. Incorporation of foreign genes was confirmed by initial GUS staining assay followed by polymerase chain reaction analysis of *rolA*, *rolB*, *rolC* and *gusA* genes. Time course study revealed that the transgenic hairy roots grew rapidly in ½ Murashige and Skoog liquid medium with highest biomass production of 0.61 g per 50 ml dry weight after 28 days of culture. In the precursor feeding experiment, the biomass of hairy roots was further increased to 0.698 g per 50 ml dry weight when 0.5 mM phenylalanine was added to the 10 days old growing culture. High Performance Thin Layer Chromatography showed that the transgenic hairy roots accumulated enhanced level of wedelolactone (422.01 μg g^{-1} dry weight), which was 1.43 and 1.37 times higher than those of *in vivo* (294.44 μg g^{-1} dry weight) and *in vitro*-grown (308.28 μg g^{-1} dry weight) plantlets, respectively. Precursor feeding with phenylalanine further augmented the accumulation of wedelolactone to 440.33 μg g^{-1} dry weight in the transgenic hairy roots. In addition, the content of several important phenolic and flavonoid compounds were increased in the transgenic hairy roots as analyzed by High Performance Liquid Chromatography. The protocol offers a scope to introduce foreign genes and further regulate the production of secondary metabolites.

1. Introduction

Sphagneticola calendulacea (L.) Pruski [synonym *Wedelia chinensis* (Osbeck) Merrill] is an essential medicinal herb belonging to Asteraceae family, commonly known as Mahavringraj or Bhringraj. It is broadly dispersed in India, China, Sri Lanka, and Japan (Kirthikar and Basu, 2006). As reviewed by Meena et al. (2011), the whole plant is believed to encompass different pharmacological properties and used for treating cough, skin diseases, dermatological disorders, headache, hair loss, lice, lack of blood, strengthening the nervous system, digestive system problems. The leaves juice promotes hair growth and also used in dyeing grey hair. Decoction of the plant is used as deobstruent and provided during menorrhagia and uterine hemorrhage (Kirtikar and Basu, 1975). The phytochemical evaluation revealed that the herb contains two main compounds, wedelolactone and demethylwedelolactone (Coumestans derivatives) as its active ingredients having anti-hepatotoxic activity (Meena et al., 2011). Different *in vivo* along with *in vitro* studies deduced anti-cancerous properties of wedelolactone (Lin et al., 2007; Chen et al., 2013). Also, the juice of the herb was also reported to possess carotene, tannin, saponin, phytosterol, chlorophyll, and stigmasterol (Koul et al., 2012). These properties are accountable for the increasing demand for *S. calendulacea* in the pharmaceutical industries. The large scale propagation of his herb has been standardized by our group (Kundu et al., 2017); however, the enhancement in the production of secondary metabolites is the matter of research.

Among the various biotechnological systems, *Agrobacterium*

Abbreviations: AS, acetosyringone; DW, dry weight; FW, fresh weight; HR, hairy root; MS, Murashige and Skoog; PGR, plant growth regulator; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography

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rhizogenes-mediated hairy root (HR) culture have gained attention in favor of secondary metabolite enhancement due to its faster growth and its ability to be cultivated in a simple medium without phytohormones (Perassolo et al., 2017). The foremost benefit is that, the equivalent or superior biosynthetic ability for secondary metabolite production is exhibited by the HRs in comparison to the mother plant (Kim et al., 2002). Yet in cases, like, *Camptotheca acuminate,* where aerial part of the plant accumulates secondary metabolite, the same metabolite was found to be present in the HR cultures as well (Lorence et al., 2004). T-DNA region of Ri (root inducing) plasmid in this bacterium comprises of *rol* genes which encodes for auxin and cytokinin biosynthesis that are accountable for HR induction (Christe and Braun, 2005) and secondary metabolite enhancement (Bulgakov, 2008). Furthermore, *A. rhizogenes* can also bear a binary vector enclosing a foreign gene and transfer into the plant genome along with the T-DNA of Ri plasmid, thus producing transformed HR expressing the target compound at a higher level (Sharafi et al., 2013). Transgenic HR is a practical model system to examine the biosynthesis of metabolites and to modify its accumulation content (Kim et al., 2010; Mehrotra et al., 2013). In addition, enhancing the production of active ingredients by precursor feeding has been conducted in a variety of plants (Rahimi et al., 2011; Ahlawat et al., 2014).

No research has been performed so far on genetic transformation of *S. calendulacea* with *A. rhizogenes*. Also, to facilitate the introduction of foreign genes into plants to enhance the amount of target compounds, conditions for transformation must be optimized. Hence, the main objective of the present investigation was to establish a stable protocol for the transgenic HR culture for enhanced wedelolactone production in *S. calendulacea*. Also, the effect of precursor feeding to further augment the wedelolactone content was investigated.

2. Materials and methods

2.1. Plant material and in vitro propagation

The *in vitro* culture of *S. calendulacea* was raised following the procedure standardized by Kundu et al. (2017). The shoots were multiplied in the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.2 mg l^{-1} thidiazuron (Sigma-Aldrich®, Bangalore, India), 0.05 mg l^{-1} naphthalene acetic acid (Sigma-Aldrich®), 30 g l^{-1} sucrose (Merck, Mumbai, India) and 8 g l^{-1} agar (HiMedia®, Mumbai, India). The cultures were maintained at 16 h photoperiod with 50 µmol $m^{-2}s^{-1}$ light intensity and 25 ± 2 °C. Nodes, internodes and leaves were aseptically excised from the *in vitro* culture and used as explants for HR induction.

2.2. Bacterial culture

The *Agrobacterium rhizogenes* strain LBA1334 harboring the natural *Ri* plasmid and a recombinant binary vector pCAMBIA1391Z consisting of hygromycin phosphotransferase (*hptII*) and *gusA* (*β*-glucouronidase) genes controlled by CaMV 2 × 35S promoter (pCAM:2 × 35S:gusA; Chattopadhyay et al., 2011), was employed for the transformation. The strain was kindly provided by Dr. Mrinal Kumar Maiti (Associate Professor, Department of Biotechnology and Advanced Laboratory for Plant Genetic Engineering, Advanced Technology Development Center, Indian Institute of Technology Kharagpur, Kharagpur 721302). The bacterial strain was cultured in Yeast Mannitol Broth medium fortified with 10 µM acetosyringone (AS), 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin (Sigma-Aldrich®). The bacterial culture was incubated overnight at 26 ± 2 °C in continuous shaking (150 rpm). Centrifugation of the bacteria cells was carried out at 10,000 rpm for 10 min and re-suspended at a cell density of A_{600} = 0.8 in liquid MS medium containing 10 µM AS, which was then applied for the transformation experiments.

2.3. Establishment of transgenic HR cultures

Excised nodes, internodes and leaves obtained from the one-month old *in vitro* culture of *S. calendulacea* were wounded by gentle pricking (approximately 20 pricks per explant) with a sterilized needle and immersed in a suspension of *A. rhizogenes* for various durations (0, 5, 10, 15, and 20 min). The infected explants were blotted dry and co-cultivated for 0, 2, 3, 4, and 5 days on Petri plates enclosing ½MS semi-solid medium, supplemented with AS (100 µM), devoid of any plant growth regulator (PGR). Each plate hold six explants and there were five Petri plates per treatment (explants) which were placed in the dark chamber at 25 °C ± 2 °C. To eliminate the bacteria following co-cultivation, the explants were rinsed with sterile water added with 500 mg l^{-1} cefotaxime (Sigma-Aldrich®) and subcultured on selection medium containing ½MS nutrients, 500 mg l^{-1} cefotaxime (to eliminate *A. rhizogenes*) and 5 mg l^{-1} hygromycin (to eliminate non-transformed roots; Sigma-Aldrich®). The emerging putative transgenic HR from the explants was considered as an independent clone. When the roots grew to a length of one cm, they were excised (keeping a portion of explant tissue) and subcultured on a fresh medium. Subculture was carried out after every 14 days and the dosage of cefotaxime was decreased stepwise to 250 mg l^{-1}, 100 mg l^{-1} and subsequently abolished from the medium. Subsequent to 42 days in semi-solid medium, individual putative transgenic HR clones were excised and cultured in 50 ml of PGR-free ½MS liquid medium taken in 150 ml conical flasks and maintained in an orbital shaker (130 rpm) at 25 ± 2 °C in dark.

2.4. Transgenic HR selection via GUS staining analysis

Histochemical GUS assay was performed for the identification of transgenic HR by submerging the tissues in GUS assay buffer solution (100 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100, 1 mg ml^{-1} X-gluc, 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide) for 12 h at 37 °C (Park et al., 2011). Subsequent to staining, the tissues were repeatedly washed with 70% ethanol and scrutinized for the detection of blue colorization.

2.5. Transgenic HR selection via molecular analysis

2.5.1. DNA isolation

Extraction of genomic DNA from the putative transgenic HRs and non-transgenic wild roots (negative control) was carried out following the CTAB method (Doyle and Doyle, 1990). Plasmid DNA was isolated from the bacterial strain (positive control) employing the rapid boiling method of Holmes and Quigley (1981).

2.5.2. Primers used for screening of transgenic HRs

Putative transgenic HRs were verified for the presence of *rolA, rolB, rolC* (derived from Ri plasmid) and *gusA* genes (derived from pCAM:2 × 35S:gusA) via sequence specific primers. To confirm the elimination of bacteria from the HR cultures, PCR amplification was performed using primers specific for *virD1* gene, located outside the T-DNA. All the primers were obtained from Xcelris Labs Ltd, Ahmedabad, India.

rolA gene: For-5′-CGTTGTCGGAATGGCCCAGACC-3′/Rev-5′-CGTAGGTCTGAATATTCCGGTCC-3′

rolB gene: For-5′-ACTATAGCAAACCCCTCCTGC-3′/Rev-5′-TTCAGGTTTACTGCAGCAG GC-3′

rolC gene: For-5′-TGTGACAAGCAGCGATGAGC-3′/Rev-5′-GATTGCAAACTTGCACTCGC-3′

gusA gene: For-5′-CACGCGTCTGTTGACTGGCAG-3′/Rev-5′-AGTTTCCCCGTTGACTGCCTC-3′

virD1 gene: For-5′-TGTCGCAAGGCAGTAAG-3′/Rev-5′-CAAGGAGTCTTTCAGCATG-3′