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Induction of hairy roots in *Gmelina arborea* Roxb. and production of verbascoside in hairy roots

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Abstract

Seedling tissues of *Gmelina arborea,* a medicinally important tree species, were infected with wild type *Agrobacterium rhizogenes* strain ATTCC 15834, which led to the induction of hairy roots from 32% of the explants. Transgenic status of the roots was confirmed by PCR using *rol*B specific primers, and subsequently, by Southern analysis of the PCR products. Six transformed clones of hairy roots were established, some of which differed in their root morphology. Doubling time of the faster growing hairy root lines was about 7 days and these cultures showed about seven-fold increase in biomass at the end of 4 weeks as compared to non-transformed seedling roots. The hairy roots showed an ability to synthesize verbascoside, a phenylpropanoid glycoside of medicinal value. This is the first report on the induction of hairy roots in *G. arborea*.

Keywords: *Gmelina arborea*; *Agrobacterium rhizogenes*; Hairy roots; *rol*B; Verbascoside; PCR

1. Introduction

Gmelina arborea Roxb. is an important timber-yielding tree from the family Verbenaceae and is naturally distributed in the moist deciduous forests of South East Asia (5–30°N latitude; 70–110°E longitude). It is also valued for its medicinal properties. Almost all parts of this tree are used in folk medicine for treating various stomach disorders, fevers and skin problems [1]. Roots of *Gmelina* are used in commercial ayurvedic preparations [2]. The plant extracts are reported to exhibit anti-inflammatory and wound healing properties [3] and are also known to inhibit platelet aggregation [4]. Chemical constituents of *Gmelina* include lignans [5], flavonoids [6], iridoid and phenylpropanoid glycosides [7] and an isoxazole alkaloid [8]. However, the active principle(s) involved in the diverse medicinal properties have not been identified.

Genetic transformation of plants using the natural vector system of *Agrobacterium rhizogenes*, the causative agent of hairy root disease in several plants, has emerged as an important alternative to intact plants as well as cell suspension cultures for the production of secondary metabolites [9,10]. Hairy roots have been reported to yield higher amounts of secondary metabolites than cell suspension cultures and in some cases, intact plant roots [11]. Growth of hairy roots can be scaled up using bioreactors and hence they can be exploited for commercial production of secondary metabolites [12]. Hairy roots of some plants are also known to produce novel secondary metabolites that are not normally present in plants, and therefore, represent a possibility for the discovery of new biologically active compounds [13–15]. Reports on genetic transformation of tree plants are limited due to their recalcitrance and long life cycle. In this paper, we report for the first time, successful establishment of hairy root cultures and production of verbascoside in an important medicinal plant *G. arborea*.

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2. Materials and methods

2.1. Plant material

Seeds of *G. arborea* were collected from the forests in Chattisgarh state, India. The seeds were removed from the stony endocarp and surface sterilized first by immersing them in 1% bavistin (Carbendazim, BASF, India) solution containing two drops of Tween 20 for 2 h. After rinsing them once with 70% ethanol, the seeds were treated with 0.1% mercuric chloride for 10 min and finally rinsed four times with sterile distilled water. The seeds were germinated on modified MS [16] medium, which consisted of half strength MS macro elements (with complete omission of NH_4NO_3 and addition of full strength $CaCl_2{\cdot}H_2O$), full strength MS minor salts and organic supplements and 1.5% (w/v) sucrose [17] for germination.

2.2. Bacterial strain and culture conditions

Wild type *A. rhizogenes* ATCC 15834 (harboring pRi 15834) obtained from IMTECH, Chandigarh, India, was used for hairy root induction. The bacteria were maintained on nutrient agar medium [(peptone 5 g l^{-1}, beef extract 1.5 g l^{-1}, NaCl 5 g l^{-1}, yeast extract 1.5 g l^{-1} and agar 15 g l^{-1}), Himedia Laboratories, India]. A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (80 rpm) at 26 °C for 16 h till the OD_{600} was about 0.5. The bacterial suspension was centrifuged at 8000 rpm for 10 min and the pellet was resuspended in 5 ml MS liquid medium and used for co-cultivation of explants.

2.3. Induction and establishment of hairy root cultures

Different seedling parts including root, stem, leaf, hypocotyl, cotyledons, cotyledonary nodal segments and embryo axis were isolated from in vitro grown seedlings and were precultured for 2 days on MS basal medium. Cotyledons were isolated after 5, 15–20 and 25–30 days from the in vitro grown seeds. The explants, which measured about 1 cm in length were removed from the medium and placed for 30 min in conical flasks containing the bacterial suspension, after which they were blotted and transferred to the modified MS basal medium mentioned in the previous paragraph. After 3 days, these explants were transferred to MS medium containing 400 mg l^{-1} cefotaxime so as to kill the residual *Agrobacterium*. Controls consisted of explants treated similarly except that they were not co-cultivated with *A. rhizogenes*. Cefotaxime concentration was then halved each week from 400 to 50 mg l^{-1}, and finally cultures free of *A. rhizogenes* were transferred to B5 [18] medium solidified with 0.2% Phytagel (Sigma). Hairy roots, which arose mainly from the cut surfaces of the explants were separated from the explants, when they attained a length of 4–5 cm and placed on B5 medium for further growth. All the cultures were maintained in complete darkness at 25 ± 2 °C. Excised roots of in vitro germinated seedlings were cultured similarly and served as controls. Six different hairy root lines were established, each line representing the occurrence of an independent transformation event. These lines were maintained by subculture of 3–4 cm long pieces on B5 solid medium after 4 weeks. The hairy root cultures were also maintained on B5 liquid medium on a rotary shaker (80 rpm) in complete darkness.

2.4. PCR analysis of hairy roots

DNA was extracted using the CTAB method [19] from each hairy root line as well as from control non-transformed roots (in vitro germinated seedling roots). PCR primers were used for amplification of a 780 bp fragment of the *rol*B gene. The sequence of each primer was as follows (forward primer 5′-A T G G A T C C C A A A T T G C T A T T C C C C C A C G A-3′ and reverse primer 5′-T T A G G C T T C T T T C A T T C G G T T T A C T G C A G C-3′). The PCR reactions were carried out in a total 50 μl volume and consisted of 200 ng of DNA, 10 pm/μl primer, 200 μM dNTP, 1 U of Taq DNA polymerase, 1 × PCR buffer and 2 mM $MgCl_2$. PCR conditions were 94 °C for 5 min (initial denaturation), 42 cycles of 94 °C for 1 min, 52.5 °C for 1.5 min and 72 °C for 2 min and a final extension at 72 °C for 10 min.

2.5. Southern analysis of PCR products

The PCR products separated on agarose gels were transferred to nylon membrane (Hybond N^+, Amersham, Pharmacia Biotech) by capillary transfer. The *rol*B gene amplified from *A. rhizogenes* using the same primers was radiolabeled with α^{32}P dCTP using a random primer kit (BRIT) according to manufacture's instruction and used for Southern hybridisation. The blotting and subsequent hybridisation was carried out according to Sambrook et al. [20].

2.6. Growth kinetics of hairy root lines

The hairy root cultures of one of the hairy root lines (line 1) were grown on different liquid media to determine the medium for optimal growth. MS basal medium, modified MS medium (mentioned earlier), B5 medium, MS medium containing B5 medium vitamins and White's medium [21] were used. Growth was measured in terms of fresh weight and dry weight of the hairy roots at the end of 4 weeks. Five replicates were used for each observation. Growth rate and doubling time of the hairy root lines growing in B5 liquid medium were measured by taking fresh weights at weekly intervals for 4 weeks. Four replicates were used for each time point. Doubling time was calculated by plotting a graph of $\log_2$ fresh weight (g) versus time (days) and calculating the inverse of slope for the linear part of the curve [22].