



Culture filtrate of root endophytic fungus *Piriformospora indica* promotes the growth and lignan production of *Linum album* hairy root cultures

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ABSTRACT

The effect of addition of autoclaved and filter-sterilized culture filtrate of *Piriformospora indica* (a root endophytic fungus) to the growing *Linum album* hairy root cultures on growth and lignan production was investigated. The addition resulted in a significant enhancement in lignan production and growth. The podophyllotoxin and 6-methoxypodophyllotoxin (the lignans) concentrations were maximally improved by 3.8 times (233.8 mg/L) and 4.4 times (131.9 mg/L) in comparison to control cultures, respectively, upon addition of 3.0% (v/v) filter-sterilized culture filtrate of *P. indica* to the hairy root cultures of *L. album* for exposure time of 48 h. This increase in the lignan content also coincided with the increase in phenylalanine ammonia lyase activity, which was 3.1-fold (371.4 μ kat/kg protein) higher compared to control cultures under the same conditions. The maximal increase in hairy root biomass was, however, obtained under different conditions; it was enhanced by 1.4 times (21.8 g/L) in comparison to control cultures, when 2% (v/v) filter-sterilized culture filtrate was in contact with *L. album* cultures for 96 h.

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1. Introduction

Plants synthesize a large number of secondary metabolites which can be used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides and food additives. Despite a number of advances in synthetic organic chemistry, the structural complexity of most of the secondary metabolites makes their chemical synthesis difficult. Therefore, plants remain an important source for commercial production of these valuable secondary metabolites as many of them are unique to plant kingdom [1]. Large demand of podophyllotoxin (a starting material for synthesis of anticancer drugs like etoposide, teniposide, and etopophose) and other phytochemicals has resulted in overexploitation of their natural sources which might lead to extension of the plant species. Therefore, there is a need of alternative methodology for large scale production of podophyllotoxin. Plant cell technology provides an alternative to the extraction of podophyllotoxin under controlled conditions. Cell cultures of *Linum* spp. have been reported to produce lignan [podophyllotoxin (PT) and 6-methoxypodophyllotoxin

(6-MPT)] with high productivities in comparison to cell cultures of other plants [2].

Combined application of high-yielding cell line selection along with other yield enhancement strategies can develop techniques for large scale production of this commercially important compound by plant cell cultures. *Agrobacterium*-mediated transformation of plant tissue cultures is one of the widely used strategies for improved production of secondary metabolites. Normal cultures generally require exogenous addition of phytohormones and have a tendency to lose their biosynthetic capability after a short period. On the other hand, *Agrobacterium*-transformed hairy root cultures are comparatively fast growing and genetically more stable, and hence are more promising for large scale production of valuable plant-specific metabolites [3,4]. Secondary metabolite synthesis and accumulation in plant cell cultures can be stimulated by the application of elicitors. Elicitors are the signaling molecules which can enhance the formation of secondary metabolites in cell cultures by inducing plant defense, hypersensitive response and/or pathogenesis related proteins [5]. Elicitors are of two types, biotic and abiotic. Among biotic elicitors, fungal elicitors have resulted in significant enhancement in the production of phytochemicals in plant tissue cultures [6,7].

Piriformospora indica, a root endophytic fungus isolated from the soil of Rajasthan (India), functions as a plant growth promoter and biofertilizer in nutrient-depleted soils. It also acts as a bioprotector against a variety of pathogens and insect invaders [8]. The fungus has shown growth promoting and yield enhancement activities on a variety of host plants. In addition to fungal biomass, the culture filtrate of *P. indica* has also shown growth-promoting effects on a

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number of plants such as maize, tobacco and *Bacopa monnieri* [9,10]. It was, therefore, interesting to investigate if *P. indica*, a known endophytic fungus with mutual synergism with plant roots, will have a beneficial effect on increasing the productivity of lignans in hairy roots cultures of *Linum album*. Hence, the present study was designed to investigate the elicitation potential of culture filtrate of *P. indica* in hairy root cultures of *L. album* for enhanced synthesis of lignans. Furthermore, compounds produced during different phases of fungal growth might differ in terms of number of elicitor moieties, chemical nature and elicitation potential. Therefore, the culture filtrates from two different phases of growth of *P. indica*, i.e., log phase (4th d) and decline phase (8th d) were used in the present study.

2. Materials and methods

2.1. Maintenance and growth of *P. indica*

The culture of *P. indica* was obtained from Prof. Ajit Varma (Amity Institute of Herbal and Microbial Studies, Noida, India). The stock culture was maintained on slants containing Kaefer medium [11] supplemented with 15 g/L agar. The slants were inoculated, incubated at $30 \pm 1^\circ\text{C}$ for 10 d and then stored at $4 \pm 1^\circ\text{C}$. For the preparation of inoculum, *P. indica* was initially grown on Kaefer medium on a petri dish and then transferred to the seed culture medium by punching out 8 mm agar discs with a sterilized cork-borer. The seed culture was grown in a 500 mL flask containing 100 mL of potato dextrose broth at $30 \pm 1^\circ\text{C}$ and initial pH of 6.5 on a rotary shaker (Scigenics, India) at 200 rpm for 4 d. For the establishment of growth kinetics, submerged cultivation of *P. indica* was carried out in 500 mL Erlenmeyer flasks containing 100 mL of Kaefer medium. The flasks were inoculated with 5 mL of freshly prepared seed culture and kept at $30 \pm 1^\circ\text{C}$ under constant shaking at 200 rpm on a rotary shaker. The flasks, in triplicate, were harvested every day till 8th d and analyzed for pH, dry cell weight and residual glucose concentration.

2.2. Development and maintenance of hairy root cultures of *L. album*

Agrobacterium rhizogenes LBA 9402 – mediated genetically – transformed high yielding hairy root line of *L. album* was developed from cotyledon segments of aseptically germinated seeds [12]. The root line was maintained by transferring 2.5 g of fresh root weight every 12th d in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg liquid medium [13] supplemented with 30 g/L sucrose on a gyratory shaker at 60 rpm and $25 \pm 1^\circ\text{C}$ under 16/8 light/dark photoperiod [12]. Hairy roots (12 d old) were used as inoculum to develop the suspension cultures for elicitation experiments. For the establishment of growth and production kinetics, the hairy roots were grown in Gamborg medium, containing 32.5 g/L sucrose and 0.75 g/L calcium chloride, with an inoculum of 5 g/L on dry weight basis. The fresh weight used for inoculation was calculated from the ratio of the fresh weight to dry weight, which was equal to 12. The cultures were incubated on a gyratory shaker at 125 rpm and $25 \pm 1^\circ\text{C}$ under 16/8 h light/dark regime [12]. The intensity of the light used in all the experiments was 1200 lx. The flasks, in triplicate, were harvested at an interval of 2 d till 16th d and analyzed for dry cell weight, lignan (PT and 6-MPT) content, residual sucrose and reducing sugars (glucose and fructose).

2.3. Elicitor preparation and its addition to *L. album* cultures

The cultures of *P. indica* were grown as described above and were harvested in mid log and decline phase i.e., on 4th d and 8th d, respectively. The fungal biomass was separated by passing through a filter paper, and the spent medium was centrifuged at $5000 \times g$ for 15 min to remove suspended particles and filtered through Whatman No. 1 filter paper. The clear solution so obtained was designated as culture filtrate and stored at $4 \pm 1^\circ\text{C}$ for further use. The culture filtrate was divided into two parts: one part was autoclaved at 15 psig for 20 min and designated as autoclaved culture filtrate while another part was filtered through 0.22 μm membrane filter and designated as filter-sterilized culture filtrate.

The two fungal elicitor preparations were added at different concentrations (0.5, 1.0, 2.0, 3.0, and 5.0% v/v) on 8th, 10th and 12th d to the growing *L. album* hairy root cultures. The culture conditions for growing *L. album* hairy roots in shake flasks remained the same as described above. The cultures were harvested on 14th d and analyzed for dry cell weight, lignan (PT and 6-MPT) content and phenylalanine ammonia lyase (PAL) activity.

2.4. Measurement of dry cell weight and residual sugars

The growth of *P. indica* was expressed in terms of dry cell weight (DCW) per liter of culture broth, which was determined by filtering a known volume of culture broth through Whatman No. 1 filter paper, drying to a constant weight in vacuum oven at $60 \pm 1^\circ\text{C}$ for about 48 h and weighing the dry weight. In case of hairy roots, DCW was determined by drying the hairy roots on Whatman No. 1 filter paper at $25 \pm 1^\circ\text{C}$ until a constant weight was achieved [4,14].

Glucose and fructose were estimated by 3,5-dinitrosalicylic acid (DNS) method [15]. For this 1.0 mL of sufficiently diluted sample was mixed with 1.0 mL of distilled water. Thereafter 3.0 mL of DNS solution was added and mixed properly by gentle vortexing. The resulting solution was boiled for 5 min on boiling water bath. Then it was cooled and 15 mL of distilled water was added to the tube and was shaken properly. Finally optical density of the solution was measured at 540 nm with respect to substrate blank. For the estimation of residual sucrose, 1.0 mL of sufficiently diluted sample was mixed with 0.5 mL of 2 N HCl, boiled for 10 min on a boiling water bath and then cooled. After cooling, 0.5 mL of 2 N NaOH was added to it and then DNS method was followed.

2.5. Extraction and analysis of lignans

The lignans, PT and 6-MPT were extracted by sonicating the dried cell mass (100 mg) with 5 mL of methanol for 15 min at $4-6^\circ\text{C}$. The supernatant was removed by centrifugation and then evaporated to dryness. The extract was redissolved in methanol and filtered through 0.22 μm filter. The extract, so obtained, was analyzed by high performance liquid chromatography (Agilent Technologies HP 100) on C₁₈ column (Waters, USA) (250 $\times$ 4.6 mm). The simultaneous separation and analysis of lignans were carried out with acetonitrile:phosphoric acid (0.01%) in water (72:28) as mobile phase at a flow rate of 0.8 mL/min and 290 nm wavelength [14]. The concentrations of the compounds were calculated by comparing the ratio of the area under the peak for the sample and the corresponding standards (PT was obtained from Sigma while 6-MPT was obtained as gift from Dr. A. Baldi of Shri R.M.S. Institute of Science & Technology, Mandsaur, India).

2.6. Phenylalanine ammonia lyase (PAL) enzyme assay

PAL enzyme was extracted from fresh hairy roots using borate buffer (0.1 M, pH 8.0) containing 5.0% (v/v) glycerol and 50 mM β -mercaptoethanol. For activity assay, the conversion of phenylalanine to *trans*-cinnamic acid was measured at 290 nm. PAL enzyme activity was expressed as μkat (μmoles of cinnamic acid formed per second) per kg protein [14].

2.7. Total protein estimation

For estimation of total protein, fresh hairy roots were powdered with liquid nitrogen using pre-cooled mortar and pestle. The proteins were extracted by homogenizing 300 mg of the powdered roots in cold 0.05 M Tris buffer. Polyvinyl pyrrolidone (0.05 g) was then added to each sample during the homogenization procedure. The homogenates were transferred to pre-cooled centrifuge tubes and centrifuged at $10,000 \times g$ for 30 min at $4 \pm 0.5^\circ\text{C}$. After centrifugation, 0.1 mL of each supernatant sample was transferred to assay tubes and 3 mL of the Bradford reagent (containing the dye) was added to each tube. Finally the absorbance of the developed color was recorded at 595 nm for total protein estimation using bovine serum albumin as standard [16].

3. Results and discussion

3.1. Growth, substrate consumption and pH profiles of *P. indica*

The growth, substrate consumption and pH profiles of *P. indica* in Kaefer medium were established and are shown in Fig. 1. After inoculation, initially a lag phase of 24 h was observed due to adaptation of fungal cells to the culture environment. Thereafter, logarithmic growth was observed for next 4 d resulting in maximum dry cell weight of 13.5 g/L on 5th d of cultivation. The fungal growth then declined due to complete consumption of glucose and no clear demarcation was observed between stationary and decline phases. The uptake of glucose caused a decrease in pH of fermentation broth during the initial growth phase which might be due to generation of acidic metabolites. However, after 2 d, it remained almost constant and started increasing after 7 d.

3.2. Growth of and lignan synthesis in hairy roots of *L. album*

The time course of growth of *L. album* hairy roots, lignan accumulation and substrate consumption are shown in Fig. 2. A maximum biomass of 15.4 g/L with concomitant sucrose consumption of 24.0 g/L was achieved on 14th d of cultivation. In addition to sucrose, the culture broth was also analyzed for the presence of glucose and fructose. However, no reducing sugar (glucose or fructose) was found. The absence of glucose and fructose might be due to low invertase activity of the plant cells. The pH increased

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