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Research article

In vitro isolation, elicitation of psoralen in callus cultures of *Psoralea corylifolia* and cloning of psoralen synthase gene

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ABSTRACT

Psoralen, an important furanocoumarin occurring abundantly in seeds of *Psoralea corylifolia* is used as an anticancerous compound against leukemia and other cancer cell lines. Evaluation and isolation of psoralen from the calluses derived from different plant parts, viz. cotyledons, nodes, leaves and roots have been done in the present case for the first time. Amongst all, a maximum of 1934.75 μg/g f.w. of psoralen was recorded in callus derived from cotyledons, followed by 1875.50 and 1465.75 μg/g f.w. of psoralen in node and leaf derived calluses, respectively. Amount of psoralen enhanced further when cotyledonary calluses were exposed to different concentrations of organic elicitors (yeast extract, proline, inositol, casein hydrolyzate (CH), glycine, glutamine and sucrose) and precursors of psoralen (umbelliferone, cinnamic acid and NADPH). Isolation of psoralen was done using methanol as solvent through column chromatography and TLC. FT-IR and NMR further characterized and confirmed the structure of psoralen. In addition, the putative gene, psoralen synthase involved in psoralen synthesis pathway has been isolated, cloned and sequenced which comprised 1237 bp length. BLAST analysis of the gene sequence of psoralen synthase revealed that its nucleotide sequence showed 93% homology with psoralen synthase isolated from *Ammi majus*. This is the first report of isolation, cloning and characterization of psoralen synthase from *Psoralea corylifolia*.

1. Introduction

Psoralea corylifolia is one of the promising medicinal plants, possessing immense biomedical applications against several diseases such as leucoderma, leprosy, psoriasis, vitiligo due to the presence of several isoflavonoids and furanocoumarins. Of the several bioactive compounds, psoralen, is one of the important furanocoumarin abundantly available in this plant which is also widely employed as an anticancerous agent against leukemia and other cancer lines [16,27,46,48].

Furanocoumarins are natural plant metabolites characterized by a furane moiety fused to benzopyran-2-one. Furanocoumarins intercalate in double-stranded DNA, and psoralens are known to cross-link pyrimidine bases under irradiation by [2 + 2] cycloaddition via their 3, 4- and 2, 3-double bonds [12]. The position of the furane substitution distinguishes two large groups of compounds, the linear (psoralens) and the angular furanocoumarins (angelicin and derivatives) [24]. Psoralen, an important furanocoumarin in particular is known for its photosensitizing and phototoxic effects and has been used in photochemotherapy of skin disorders (psoriasis, vitiligo, and mycosis). Due to the complex bioactivity of furanocoumarins, its biosynthesis has received continuous attention. Knowledge of the biosynthetic pathway of psoralen may enable us to influence its formation in a direct way, for example by metabolic pathway engineering. The biosynthetic pathways to the linear furanocoumarin (Psoralen) involved enzymes (and their cofactors) which are as follows 1. DMAPP-umbelliferone dimethylallyl transferase 2. marmesin synthase (O_2, cytochrome P450, NADPH), 3. psoralen synthase (O_2, Cyt, P450, NADPH) [5] (Fig. 1).

The basis of linear furanocoumarin formation was mostly established by the end of the 1980s by a combination of precursor feeding experiments and the biochemical characterization of major enzymes of the pathway [15]. Nevertheless, until recently, the key enzyme genes in the pathway of psoralen synthesis in *P. corylifolia* had not yet been cloned and sequenced.

The present study highlights the evaluation of the psoralen content in (i) calluses derived from different plant parts of

Abbreviations: $CDCl_3$, deuterated chloroform; DMAPP, dimethylallyl pyrophosphate; FT-IR, Fourier transform infra red; F.W., fresh weight; IPTG, isopropyl-β-thio galactopyranoside; $LiTaO_3$, lithium tantalate; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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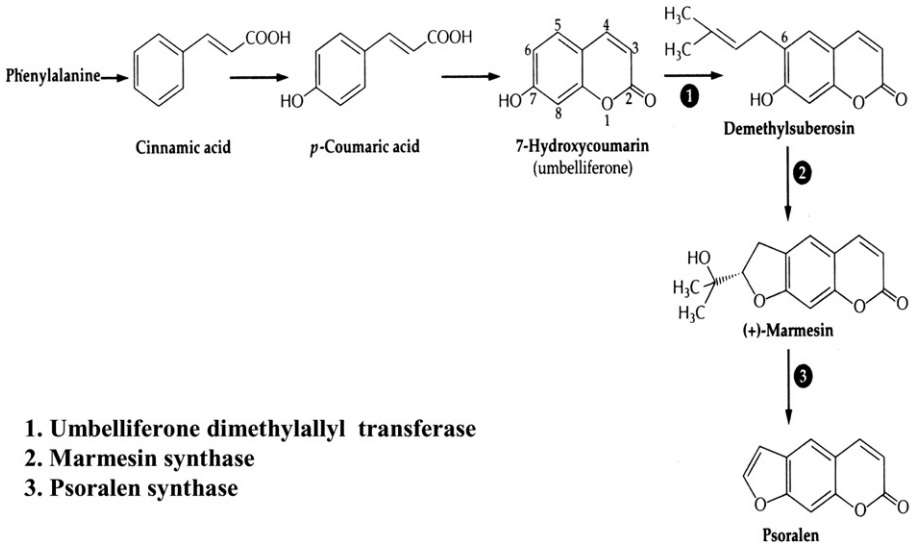

Fig. 1. Schematic representation of the biosynthetic pathway of psoralen. (Source: Croteau et al., 2000 [5]).

P. corylifolia, *viz*. cotyledons, nodes, leaves and roots (ii) *in vitro* elicitation of psoralen employing organic elicitors and precursors of the psoralen biosynthetic pathway (iii) isolation and characterization of psoralen from callus and (iv) isolation and characterization of putative gene, psoralen synthase involved in psoralen biosynthetic pathway.

2. Materials & methods

2.1. Plant materials

The seeds of *P. corylifolia* were procured from Homeopathic Pharmacopoeia Laboratory, Ghaziabad (Uttar Pradesh, India) and were sown in the seed beds of Botanical Garden, Department of Botany, University of Delhi, in the month of March. The leaf, node, root and green seeds of *P. corylifolia* were taken from field grown mature plants. These explants were washed thoroughly under running tap water for 20 min and treated with 1% bavistin (w/v), for 10 min with constant vigorous shaking on rotary table top shaker at 150 rpm, to provide better surface contact with the fungicide. These were rewashed under running tap water to remove any traces of bavistin. After pouring out excess of water, the explants were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 2 min and finally washed 4 or 5 times with sterilized distilled water.

2.2. Culture media

MS (Murashige and Skoog, 1962) [23] and B5 (Gamborg et al., 1968) [8] media were employed for raising the cultures of *P. corylifolia*. Analytical grade (AR) salts (Qualigens or Glaxo Fine Chemicals, Mumbai) were used to prepare the stock solutions. The basal medium was supplemented with various growth regulators such as N^6-benzyladenine (BA), α-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) (Sigma–Aldrich, USA), organic compounds (Casein hydrolyzate, Glycine, Myo-Inositol, Glutamine, Proline, Yeast extract in the range of 0, 1, 5, 25, 50, 100, 200 & 300 mg/l and Sucrose; 0, 1.5, 3, 4.5, 6, 7.5 & 9%) and precursors of psoralen (Cinnamic acid, NADPH and Umbelliferone; 0, 0.1, 1, 2.5, 5, 25 & 50) (Sigma Aldrich, USA). As a source of carbon, 3% (w/v) sucrose (Daurala, DCM, Meerut) was used in all experiments unless mentioned specifically.

The media were gelled with 0.8% agar (Qualigens Mumbai) and the pH of media was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving. Approximately, 20 ml media was dispensed in each 2.15 × 15 cm test tubes (Borosil) plugged with non-absorbent cotton wrapped in muslin cloth and was autoclaved at 1.06 kg cm^{-2} at 121 °C for 15 min.

2.3. Raising and incubation of cultures

Cultures were incubated in continuous light (28–35 μmol m^{-2} s^{-1}) by cool day light fluorescent incandescent tubes (40 W, Philips, Kolkata). The cultures were maintained in a culture room at the temperature of 25±2 °C and 55 ± 10% relative humidity. The explants were sub-cultured after every 30–32 d interval on the same but fresh medium. Observations were recorded at an interval of seven days. The final data were recorded after 30 d of inoculation.

2.4. Statistical analysis

The evaluation of psoralen from various callus samples of *P. corylifolia* was performed with four replicates each and the data obtained were analyzed statistically. The statistical analyses were performed by ANOVA using SPSS. The differences between means were tested for significance by Duncan's multiple range test at $p = 0.05$.

2.5. Phytochemical analysis

The fresh samples (1 g, each) of different plant tissue were crushed with liquid nitrogen and soaked in ethanol for 24 h, under dark and then homogenized using pestle and mortar. After evaporation of ethanol, the semisolid form of extract was mixed in methanol (HPLC grade). This mixture was transferred to centrifuge tube and centrifuged for 15 min at 12,000 rpm at room temperature. The pellet was discarded and the supernatant was filtered using 0.22 μm millipore filter for further analysis. The HPLC unit of Shimadzu-4A type, equipped with UV detector and printer plotters was operated under the following parameters: Column: C18; Column packing: Zorbex ODS (Octadecyl silane); Solvent: Methanol (HPLC grade); Injection volume: 20 μl; Flow rate: 0.5 ml/min; Detection: UV 244 nm for psoralen.