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Short communication

Influence of hormones and medium components on expression of dipyranocoumarins in cell suspension cultures of *Calophyllum inophyllum* L.

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ABSTRACT

Cell suspension cultures were initiated separately from leaf and nodal/internodal calluses for the study of influence of hormones and medium components on biomass growth and expression of dipyranocoumarins. Highest 6.2 times biomass was enhanced in suspension cultures of nodal/internodal callus supplemented with threefold total sulphate. Picloram 8.28 μM along with BAP 8.88 μM enhanced 295.05 times inophyllum A in suspension cultures of leaf callus whereas IBA 14.70 μM along with BAP 4.44 μM in suspension cultures of leaf callus enhanced 1065 times inophyllum B. IBA 4.90 μM alone in suspension cultures of nodal/internodal callus enhanced maximum 616 times inophyllum C. Only IBA 9.80 μM in suspension cultures of leaf callus enhanced 23.22 times inophyllum P. Variation in nitrate and sulphate had maximum positive influence on expression of inophyllums A and C and vitamins had maximum positive influence on expression of inophyllums A, C and B.

1. Introduction

Calophyllum inophyllum Linn (Clusiacease), popularly known as Alexandrian Laurel or Indian Laurel is a rich source of medicinally important bioactive compounds like xanthones [1–5], triterpenes [3,6], terpenoids [7], benzopyrano derivatives [8] and coumarins [9]. Among these, dipyranocoumarins are the most important group of bioactive compounds isolated from *C. inophyllum*. Dipyranocoumarins inophyllums B and P were reported to have anti-HIV activities and act as Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI) [10]. Eleven compounds of inophyllum class were isolated from *C. inophyllum* and were described together with the SAR (Structure–Activity Relationship) of these novel anti-HIV compounds [10]. Inophyllum C and calophyllolide also showed antimicrobial and cytotoxic activity [5]. *Calophyllum lanigerum* from which potential anti-HIV, NNRTIs calanolides were isolated and reported [11] has not been reported so far from India. As an alternative to calanolide and its source *C. lanigerum*, another NNRTIs inophyllums isolated and reported from *C. inophyllum* could be of immense help in anti-retroviral therapies. *C. inophyllum* grows on sandy beaches near the coasts along the Western Ghats of India.

As a part of our study on *in vitro* production of dipyranocoumarins in *C. inophyllum*, we recently developed and reported the methods for induction of callus cultures from different explants, their extraction and HPLC analysis [12]. Using these methods, we initiated the cell suspension cultures in *C. inophyllum*. Cell suspension culture is method of choice for secondary metabolite production. Recently suspension culture systems was successfully studied and employed for production of azadirachtin in *Azadirachta indica* [13]; antrhraquinine in *Morinda elliptica* [14]. The objectives of the present study were to initiate cell suspension cultures of leaf and nodal/internodal callus using Woody Plant Medium (WPM) [15] and to study the influence of hormones on biomass growth and expression pattern of dipyranocoumarins (inophyllums A, B, C, D, P and calophyllolide). The influence of medium components like total nitrates, total sulphates and total vitamins of WPM on biomass growth and expression pattern of dipyranocoumarins has also been discussed.

2. Materials and methods

2.1. Initiation of suspension cultures

A method for induction of callus cultures from different explants of *C. inophyllum* was recently developed and reported [12]. These callus cultures were maintained in our laboratory by sub-culturing after every 30 days and used for initiating cell suspension cultures. Two types of cell suspension cultures viz. suspension cultures of leaf callus and suspension cultures of nodal/internodal callus were initiated separately and studied. Suspension cultures were established by inoculating the 1–2 g of 2–3-month-old callus masses in 250 mL Erlenmayer flasks containing 100 mL liquid WPM. These cultures were incubated on gyratory shaker rotating at 120 rpm. The temperature and photoperiod was maintained at 25 ± 1 °C under cool white fluorescent continuous light (35 μmol m^{-2} s^{-1}; Philips, India). The sub-culturing of suspension cultures to fresh media was done after every 30 days of incubation. Each experiment was repeated 2 times.

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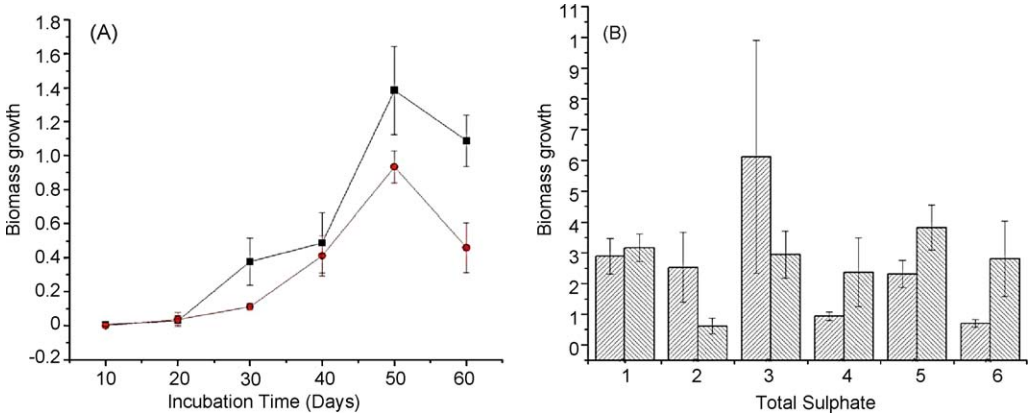

Fig. 1. Biomass growth in suspension cultures. (A) Time course of biomass growth in suspension cultures of leaf and nodal/internodal callus. (B) Effect of total sulphate on biomass growth in suspension cultures of leaf and nodal/internodal callus. Each value represents mean ± SD of four replicates of two experiments. Biomass growth in g L^{-1} FW. (■) Suspension culture of leaf callus; (●) suspension culture of nodal/internodal callus; (▨) biomass growth in suspension cultures of leaf callus; (▧) biomass growth in suspension cultures of nodal internodal callus. 1: WPM − sulphate; 2: WPM (control), 3: WPM + 2 × sulphate; 4: WPM + 3 × sulphate; 5: WPM + 4 × sulphate; 6: WPM + 5 × sulphate.

2.2. Study of growth kinetics

Effect of different incubation period (no. of days) on growth of biomass and expression pettern of dipyranocoumarins in suspension cultures of both callus types was studied to optimize incubation period required for maximum biomass growth and expression of dipyranocoumarins. For the study of growth kinetics, suspension cultures were established in liquid WPM without any phytohormones and medium component manipulations. Suspension cultures were harvested after every 10 days up to the period of 60 days for estimation of biomass growth and extracted for HPLC analysis.

2.3. Medium manipulations

To investigate the influence of hormones on biomass growth and expression pattern of dipyranocoumarins, masses of both callus types were inoculated separately in liquid WPM with 2% sucrose and incorporated with IBA (4.90–19.60 μM), IBA + BAP (4.90–19.60 μM + 4.44 μM) and Picloram + BAP (8.28–33.12 μM + 8.88 μM). For studying the effects of different medium component manipulations, concentrations of total nitrate, total sulphate and total vitamins were varied in liquid WPM. From the composition of WPM basal medium, separate stocks of total nitrate (NH_4NO_3, 0.4 g/L + Ca $(NO_3)_2$, 0.556 g/L), total sulphate ($MgSO_4$, 0.370 g/L + K_2SO_4, 0.990 g/L + $MnSO_4$, 2943 μg/L + $ZnSO_4$, 8600 μg/L + $CuSO_4$, 2650 μg/L) and total vitamins (nicotinic acid, 0.5 mg/L + thymine HCl, 1.0 mg/L + pyridoxin HCl, 0.5 mg/L + glycin, 2.0 mg/L + inositol, 100 mg/L) were prepared and added. Total sulphate, total nitrates and total vitamins were varied in the range of nil–4.0-fold (nil–5×) increase. All these media combinations with varied concentrations of hormones and medium components were inoculated with leaf and nodal/internodal calluses to initiate suspension cultures of leaf and nodal/internodal calluses.

2.4. Extraction, sample preparation and HPLC analysis

Except for the study of growth kinetics, all suspension cultures were harvested and extracted between 45–50 days of incubation. Suspension cultures were filtered through Whatmann's filter paper No-1 to collect biomasses and culture filtrates. Culture filtrates were partitioned with chloroform in separating funnels. After thorough mixing, separating funnels were kept aside for 10 min to separate two immiscible solvents. Thereafter, chloroform layers were separated and concentrated. These chloroform extracts of culture filtrates were then dissolved in HPLC mobile phase and used for HPLC analysis. Remaining biomasses were weighed and biomass growth was determined by using following formula.

$$\text{Biomass growth} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}$$

After determining the biomass growth, extraction, sample preparation and HPLC analyses of biomasses were performed as described previously [12]. Standards of inophyllums A, B, C, D, P and calophyllolide were provided by Division of Organic Chemistry, National Chemical Laboratory, Pune, India. [16]. Two-way ANOVA was performed on Microsoft Excel and OrginPro 8.

3. Results and discussion

Initially we established suspension cultures with seed, leaf and nodal/internodal callus types. Biomass in suspension cultures of seed callus did not grow because of their brown, friable and necrolytic nature. Therefore, suspension cultures of seed callus were not studied further. In HPLC profiles of culture filtrates of suspension cultures, peaks corresponding to the dipyranocoumarins were not detected. This indicated that dipyranocoumarins were not released in the medium. In the HPLC profiles of biomass, peaks corresponding to the dipyranocoumarins were detected.

3.1. Time course of biomass growth and expression of dipyranocoumarins

The time course of biomass growth in cell suspension cultures of both callus types followed typical growth curve like pattern (Fig. 1A). In these suspension cultures, biomass did not increase till 20 days and then started growing during 20–40 days. Exponential growth of biomass was followed up to 50 days. Maximum biomass growth in suspension cultures of leaf callus (1.383 g L^{-1}) and nodal/internodal callus (0.932 g L^{-1}) was observed at 50 days incubation (Fig. 1A). Phytohormones are known to influence the biomass growth as well as production of secondary metabolites in different plant species. Accumulation of secondary metabolite like phenylpropanoids was shown to be affected by phytohormones independently from their effect on growth [17,18]. To rule out any such possibilities of hormonal interference and influence during the study of growth kinetic, cell suspension cultures were initiated in liquid WPM basal medium without any growth hormones. Compared to the herbs or shrub plant species, higher woody plant species are known to be slow in their growth. Sometimes their slow growth rate is also reflected in *in vitro* growing cultures. *C. inophyllum*, being the woody tree species was also slow in its growth rate and its *in vitro* cultures also grew slowly. This is why for maximum biomass growth in suspension cultures required incubation period up to 50 days. This results suggested that incubation period of 50 days was optimum for biomass growth in cell suspension cultures of both calluses. During the study of biomass growth kinetics, biomasses harvested at different incubation period were also extracted and analyzed with the HPLC method. HPLC analyses and overall expression pattern over the incubation period of 60 days revealed that expressions of the dipyranocoumarins were maximum during 40–50 days of incubation (complete data not shown). Therefore, it was concluded that incubation period for about 40–50 days was best suited for the maximum expression of dipyranocoumarins.

3.2. Effect of hormones on biomass growth and expression pattern of dipyranocoumarins

In suspension cultures of leaf callus, maximum 5.46 g L^{-1} (3.6 times) biomass growth was resulted in WPM with higher