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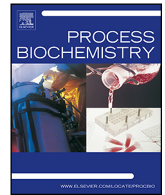

Enhanced cephalotaxine production in *Cephalotaxus mannii* suspension cultures by combining glycometabolic regulation and elicitation

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ABSTRACT

To study the combination effects of glycometabolic regulator NaF and elicitor methyl jasmonate (MJ) on cephalotaxine production in *Cephalotaxus mannii* suspension cultures, NaF of 10 mg/L, MJ of 100 μmol/L or both of them (NaF + MJ for short below) were added to the shake-flask cultures of *C. mannii* cell. It was found that NaF increased the activity of glucose 6-phosphate dehydrogenase (G6PDH), but had no significant effects on phenylalanine ammonium-lyase (PAL) activity and phenols formation. In contrast, MJ could activate PAL activity and led to phenols accumulation, but had no significant effects on G6PDH activity. To explore the effects of NaF and MJ on cephalotaxine biosynthesis, harringtonine and homo-harringtonine, the two cephalotaxines, were analyzed in this work. The results obtained indicated that NaF + MJ treatment showed the strongest promotion of production in all tests. Harringtonine yield in NaF + MJ treated cells (7.245 mg/L) was 4.8-fold higher than that in control cells (1.506 mg/L), 1.7-fold that in NaF-treated cells (4.12 mg/L) and 1.6-fold that in MJ-treated cells (4.458 mg/L), respectively. No homoharringtonine was found besides in NaF + MJ treated cells (0.491 mg/L). With respect of the product release rates, they were 0%, 78%, 24% and 62% in control, NaF, MJ and NaF + MJ treatment, respectively. These results suggest that the combination of NaF and MJ had contributed to the synthesis and secretion of cephalotaxine in *C. mannii* cells.

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1. Introduction

Cephalotaxus mannii, a plant indigenous to Hainan China, can produce a number of valuable anti-cancer ester-type cephalotaxus alkaloids (*e.g.*, harringtonine and homoharringtonine, *etc.*) for treatment of different types of leukemia [1]. Since these plants are difficult to propagate and their numbers are rare, *C. mannii* had been listed as national rare and endangered plant of second-grade protection in China [2].

The plant suspension cell culture has been proposed as the most promising alternative approach to sustainable production of plant-derived drugs [3–5]. Many adopted strategies (*e.g.*, elicitor, metabolic regulation and the screening of high-growth cell, *etc.*) have been developed to increase the yield of secondary metabolites in plant cell cultures [6]. Among them, adding elicitor to plant cultures is the most effective way to promote the synthesis of plant secondary metabolites, based on the reason of its activating the gene expression of enzymes related to plant secondary metabolite synthesis through eliciting the defense response of plant cells [7–9].

Cephalotaxine (ester-type cephalotaxus alkaloids) is biosynthesized originally from one molecule each of tyrosine and phenylalanine [10,11]. The synthesis of tyrosine and phenylalanine is involved in Enbden–Meyerhof–Parnas (EMP) pathway, hexose monophosphate (HMP) pathway, shikimate pathway and phenylpropanoid pathway as shown in Fig. 1. Fig. 1 illustrates that the carbon skeletons of cephalotaxine derive from phosphoenolpyruvate (EMP pathway) and erythrose-4-phosphate (HMP pathway). In normal physiological circumstances, EMP pathway is the main metabolic pathway of glucose for plant cell growth and can supply sufficient phosphoenolpyruvate. Thus we could speculate that erythrose-4-phosphate from HMP pathway is one of restrictive factors for cephalotaxine biosynthesis. Therefore, through the metabolic flux regulation, if we can put the EMP pathway of glucose metabolism to the HMP pathway, increasing the amount of erythrose-4-phosphate, which is helpful to promote the synthesis of cephalotaxine.

Sodium fluoride (NaF), a metabolic inhibitor of the EMP pathway, can inhibit the activity of aldolase and pyruvate kinase in the

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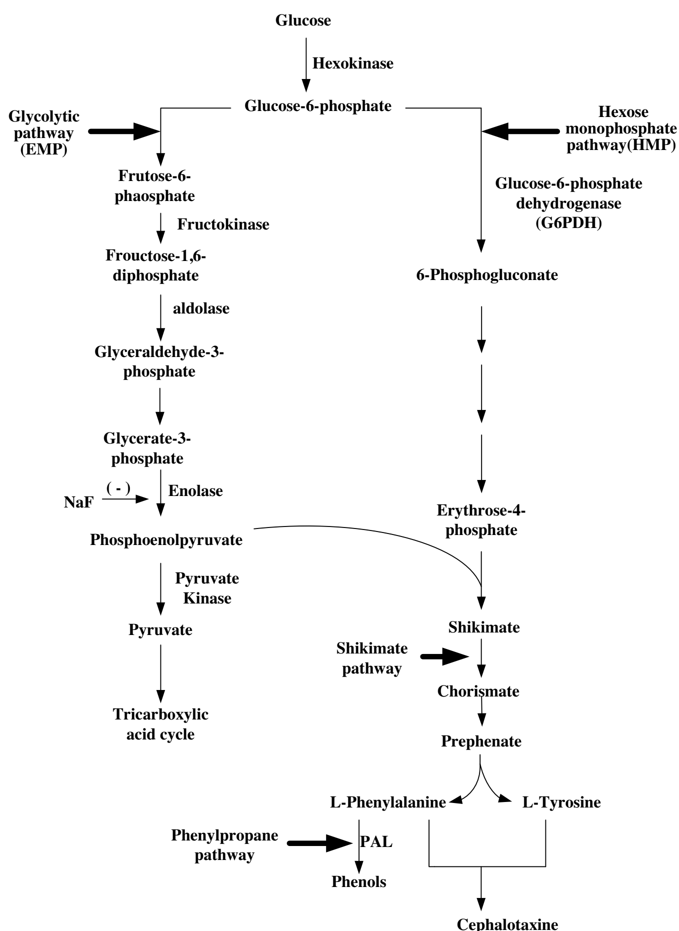

Fig. 1. Schematic illustration of biosynthetic pathway of cephalotaxus ester alkaloids.

EMP pathway [12,13], and makes carbon metabolic flux flow to HMP pathway from EMP pathway, finally, increases the amount of erythrose-4-phosphate. Glucose 6-phosphate dehydrogenase (G6PDH) is a rate-limiting enzyme in the HMP pathway, so it may influence the flux of the HMP pathway. As an elicitor, MJ can activate the gene expression of the related enzymes involved in the synthetic pathways of plant secondary metabolite. The aim of this study was to characterize the synergistic effect of NaF and MJ on cephalotaxine production and release in *C. mannii* cells. In addition, sugar consumption, the activity of phenylalanine ammonialyase (PAL) and G6PDH and phenols accumulation related to the synthesis of cephalotaxine, also was investigated.

2. Materials and methods

2.1. Plant cell culture

The plant materials were collected from wild plant populations in Bawangling national nature reserve of Hainan, China. *C. mannii* callus was induced from its bacteria-free leaves with callus induced medium contained MS salt [14], naphthalene acetic acid (NAA) 3 mg/L, kinetin (KT) 0.15 mg/L, polyvinylpyrrolidone (PVP) 0.5 g/L, casein enzyme hydrolysate 1 g/L, and sucrose 35 g/L. The callus obtained was cultured under dark condition and at 27 ± 1 °C.

C. mannii suspension cells was performed by transferring the callus solid cultures to a 250-mL Erlenmeyer flask containing 80 mL of MS liquid medium supplemented with NAA 4 mg/L, KT 0.15 mg/L, PVP 0.5 g/L, casein enzyme hydrolysate 1 g/L, and sucrose 35 g/L, growing in darkness on a gyratory shaker at 100 rpm and 27 ± 1 °C. Further subculturing was maintained by diluting cultures at the end of a growth period with fresh medium in 1:3 ratio (12-day cycle). Seed cultures were subcultured for five generations in flasks prior to test.

2.2. Metabolic inhibitor and elicitor preparations

NaF was dissolved in distilled water and sterilized before using. MJ was dissolved in absolute ethyl alcohol and filter-sterilized with a 0.22-μm membrane.

2.3. Experimental protocols

To obtain shake-flask cultures, an aliquot (15 mL) of 12-day-old subcultured suspension cultures containing approximately 6 g fresh weight (FW) cells were inoculated into 65 mL of MS liquid media described above in a 250-mL Erlenmeyer flask, then cultured in darkness on a gyratory shaker at 100 rpm and 27 ± 1 °C. At day 15 of culture period, NaF and MJ were added into the shake-flask cultures and to final dosages of NaF 10 mg/L and MJ 100 μmol/L, respectively. The cultures were harvested at day 30 of culture. PAL activity was assayed at 24 h after adding MJ or NaF into the cultures, and G6PDH activity was assayed at 48 h.

2.4. Analysis of sugars and total phenolics in cell culture media

In this document, phenol-sulfuric acid assay was used to determine the content of residual sugars in cell culture media [15]. Briefly, 1 mL culture supernatant obtained by centrifugation was mixed with 1 mL of 5% (w/v) phenol solution and 5 mL of 98% H_2SO_4, then keeping the reaction mix at room temperature for 30 min. Finally, measuring the absorbance at 480 nm and using glucose as a standard.

Total phenolics in cell culture media were analyzed by spectrophotometry as described previously [16,17]. First, two milliliters of *C. mannii* cell culture supernatant were obtained by centrifugation and then mixed with 10 mL ethyl acetate. Afterward, ethyl acetate phase was separated from the aqueous phase, after which 5 mL of ethyl acetate was evaporated at room temperature and the residue was re-dissolved in 3 mL 75% (v/v) ethanol, and then analyzed sepectrophotometrically at 280 nm using salicylic acid as a standard. Measurements obtained expressed the relative amount of total phenolics accumulated in cultures.

2.5. PAL activity assay

At 24 h after adding MJ or NaF into cultures, PAL was extracted from fresh cell mass (300 mg FW) with 2 mL of 50 mmol/L pH 8.8 Tris–HCl buffer containing 15 mmol/L of β-mercaptoethanol, and were then homogenized in an ice-cooled mortar. The homogenate was centrifuged at 10,000 × g and 4 °C for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on the PAL conversion of L-phenylalanine to cinnamic acid [18]. Briefly, 1 mL of the extraction buffer above, 0.5 mL of 10 mmol/L L-phenylalanine, 0.4 mL of double distilled water, and 0.1 mL of enzyme extract were incubated at 37 °C for 1 h. The reaction was terminated by adding 0.1 mL of 6 mol/L HCl. The absorbance of the reaction solution was measured at 290 nm. One unit (U) of enzyme activity was defined as 0.01 A_{290} increase per g fresh cell in one min.

2.6. G6PDH activity analysis

At 48 h after adding MJ or NaF into cultures, approximately 200 mg fresh cells were homogenized in an ice-cooled mortar with extraction buffer consisting of 0.1 mol/L Tris–HCl (pH7.5) and 1%

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