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Paclitaxel-producing fungal endophyte stimulates the accumulation of taxoids in suspension cultures of *Taxus cuspidate*

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ABSTRACT

Fusarium mairei, a paclitaxel-producing fungal endophyte, its effects on taxoid synthesis in *Taxus* cells were investigated via adding the fungal endophyte culture supernatants (FECS) to the suspension cultures of *Taxus cuspidata*. The concentration of FECS was determined on its total carbohydrate. When 100 mL of *Taxus* cell suspension cultures were treated with several dosages of FECS (4, 6 and 8 g) at day10, the cultures treated with 6 g FECS produced the highest yield of paclitaxel (5.84 mg L^{-1}), which was 1.8-fold the yield from controls (3.14 mg L^{-1}). The major elicitor element in FECS was an oligosaccharide of 2 kDa. In addition, the cultures treated with 6 g FECS resulted in 25.86 mg L^{-1} of the precursor 10-deacetylbaccatin III (10-DAB) accumulation, which was 11 times that of control cultures (2.32 mg L^{-1}), and 4.7 times higher than that of cultures treated with 200 μM methyl jasmonate (MJ) (5.43 mg L^{-1}). These results indicate that FECS favors to stimulate 10-DAB accumulation more effectively than paclitaxel accumulation.

1. Introduction

Paclitaxel is a potent antimitotic agent with excellent activity against a range of cancers (Suffness and Wall, 1995), but a major obstacle to its clinical use is its limited supply. Several methods have been developed for paclitaxel production. The total chemical synthesis of the drug is not considered economically feasible due to the high cost and low yield (Holton et al., 1995a; Nicolaou et al., 1994). Plant cell culture has been considered an attractive alternative. But commercialization has been hampered by its low paclitaxel yield, resulting from low gene expression of components involved in the paclitaxel biosynthesis pathway. Semisynthesis is currently an efficient route for the production of paclitaxel from its precursor, 10-deacetylbaccatin III (10-DAB), which is found in yew needles and twigs (Commercn et al., 1995; Holton et al., 1995b). Although semisynthesis procedures are efficient, they rely on the extraction of advanced taxoids (e.g. 10-DAB) from plant material that requires substantial effort to separate the desired intermediate(s) from abundant phenolics, lipids and other contaminants. Those problems may be overcome via plant cell culture process, because the isolation of taxoids from *Taxus* cultures requires fewer steps than purification from intact plant tissue.

Regardless of paclitaxel or its precursor 10-DAB are the secondary metabolites produced in *Taxus* cells. The synthesis of secondary metabolites is part of the defense response of the plant to environmental stresses and pathogen attacks. Strategies for improving the yield of secondary metabolites in plant cell suspension cultures should be based on this mechanism (Zhao et al., 2005b). Many elicitors and signal molecules, e.g. methyl jasmonate (MJ), arachidonic acid, silver ion, chitosan, La^{3+} ion and fungal mycelia extracts, have been used to improve taxoid production based on this principle (Yukimune et al., 1996; Mirjalili and Linden, 1996; Wang et al., 1999, 2001; Yukimune et al., 2000).

We have derived a paclitaxel-producing fungal endophyte from the inner bark of the China maire yew and identified it as *Fusarium mairei* (Xu et al., 2006). The ecological relationships between fungal endophytes and their respective plant hosts are very complex. A fungal endophyte may survive in a plant as a symbiont by providing protective substances (e.g. antifungal, antibacterial) that may inhibit or kill tissue invading pathogens (Strobel et al., 1997), while the plant also provides nutrients for the fungus. The roles of fungal endophytes and their plant hosts during accumulation of secondary metabolites are even more complex, and the roles of *F. mairei* and its plant host (*T.* sp.) during accumulation of paclitaxel are not known. However, the concentration of paclitaxel and the number of microorganisms in the old bark of a yew tree are higher than those in young tissues (Nadeem et al., 2002; Zhao et al., 2005a). Thus, the fungal endophyte may play an important role in paclitaxel biosynthesis in the yew tree. Here we aim to elucidate

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the effects of endophytic fungus on taxoid synthesis and its action sites of taxoid synthesis pathway in *Taxus* suspension cells

2. Materials and methods

2.1. Plant cell cultures

T. cuspidata (line T168) cells were grown in darkness on a gyratory shaker at 100 rpm and 25 ± 1 °C in a liquid medium containing naphthaleneacetic acid (NAA) 2 mg L^{-1}, 6-benzylaminopurine (6-BA) 0.15 mg L^{-1}, casein acid hydrolysate 1 g L^{-1}, sucrose 25 g L^{-1} and inorganic salt formulation of B5 (Gamborg et al., 1968). An aliquot (20 mL) of 12-day-old subcultured suspension cultures (approximately 6 g fresh weight of cells) was inoculated into 80 mL of B5 medium in a 500-mL Erlenmeyer flask. Suspension cultures of *T. cuspidata* were subcultured every 12 days for 3 generations prior to this experiment.

2.2. Maintain methods for fungal endophytes

F. mairei (strain Y117) was isolated from the inner bark of a China maire yew and maintained in the medium consisting of glucose 20 g L^{-1}, NH_4NO_3 3 g L^{-1}, $MgSO_4$ 0.5 g L^{-1} and KH_2PO_4 0.5 g L^{-1} at 25 °C

2.3. Preparation of fungal endophyte culture supernatants (FECS)

F. mairei cultures were performed by transferring the maintaining cultures into a 500-mL Erlenmeyer flasks containing 200 mL B5 medium except for plant hormones (NAA and 6-BA) on a gyratory shaker at 170 rpm and 25 ± 1 °C for 6 days. The culture broth was then filtered through 4 layers of cheesecloth, centrifuged at 10,000 × *g* for 20 min and filtered through a 0.4-μm filter. The filtrate of 90 mL was evaporated to a final volume of 30 mL under vacuum at 60 °C. This triple concentrated culture broth was sterilized at 121 °C for 15 min before use.

The concentration of FECS was determined on its total carbohydrate. The total carbohydrate was analyzed by phenol–sulfuric acid method using glucose as the standard. Briefly, a sample (1 mL) of FECS was mixed with 5 mL H_2SO_4 and set at room temperature for 30 min, and then the absorbance was analyzed at 480 nm.

2.4. PAL activity analysis

Phenylalanine ammonium-lyase (PAL) was extracted from fresh cell mass, the measurement of PAL activity was based on the PAL conversion of L-phenylalanine to cinnamic acid (Yuan et al., 2002).

2.5. Taxoid extraction and analysis

For the extraction of taxoids from *T. cuspidata* cells, 100 mg of dried cells were powdered in a glass mortar with a pestle, soaked with 20 mL of methanol for 12 h, and then mixed with 20 mL of distilled water. The mixture was extracted three times with 20 mL of trichloromethane. The trichloromethane phase was separated from the aqueous phase and evaporated to dry at 40 °C under vacuum. The remaining taxanes were re-dissolved in 1 mL methanol and filtered through a 0.2-μm filter prior to HPLC.

Extracellular taxoids were extracted from the culture medium using the same volume of trichloromethane three times.

Quantification of the taxoids was performed via a reverse-phase HPLC system (Waters 2696) with a Sunfire-C18 column (2.1 mm × 150 mm, 5 μm), and a mobile phase consisting of methanol: formic acid (0.2%, v/v) at 85:15 (v/v), flow rate, 0.3 mL/min, and detected by diode array detector (DAD) at 238 nm. Identification of the taxoids was accomplished by comparison of retention times and LC/MS fragmentation patterns with authentic standards (Sigma).

2.6. Statistics

The experimental data are reported as the mean of three independent experiments. The significance of differences among experimental points was determined by two sample paired *t*-tests. A *P* value less than 0.05 was considered to be statistically significant. Results are expressed as mean ± S.D.

3. Results

3.1. Effects of fungal endophyte culture supernatants (FECS) on the cell growth as well as paclitaxel production

The effects of FECS on the growth of *T. cuspidata* cells are illustrated in Fig. 1. It shows that the growth curve of cells treated with 4 g FECS was similar to that of the control cells. For 2 days after the addition of FECS, the specific growth rates of the plant cells were 0.08, 0.04, 0.03, 0.02 day^{-1} for the control cells and cells treated with 4, 6 and 8 g FECS, respectively. But specific growth rates of 0.15, 0.14, 0.12 and 0.1 day^{-1} were observed, respectively, during days 2–8 after treatment. These results implied that the inhibition of the growth of *T. cuspidata* cells by FECS was short-lived and could be alleviated by adaptation to FECS. No significant differences ($P > 0.05$) were observed in *T. cuspidata* cell growth using 4 and 6 g FECS treatments compared with control cells. But the cells treated with 8 g FECS displayed a significant ($P < 0.05$) decrease in growth compared with control cells. From these results it may be inferred that FECS produced no significant effects on the growth of *T. cuspidata* cells when the amount added was less than 6 g.

The effects of FECS on paclitaxel accumulation and release are shown in Table 1. The cultures treated with 6 g FECS produced the largest amount of paclitaxel (5.84 mg L^{-1}) and a release ratio of 67%, which were 1.8-fold and 5.6-fold higher than the values for the controls (3.14 mg L^{-1}, 12%), respectively.

3.2. Primary analysis of active ingredients in fungal endophyte culture supernatants (FECS)

A mixture of 300 mL pre-cooled alcohol and 100 mL of FECS was settled for 12 h at 4 °C. After being centrifuged at 10,000 × *g* for 10 min, it was divided into two parts, the supernatant and

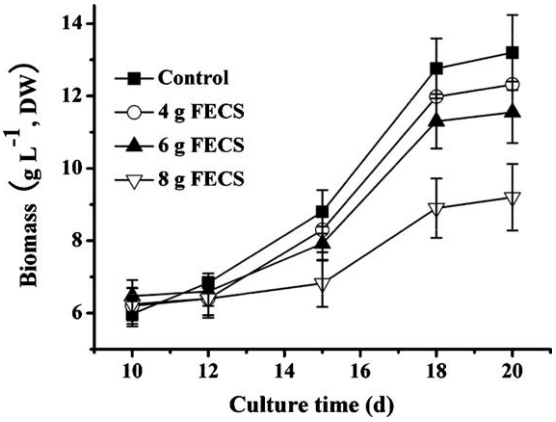

Fig. 1. Effects of fungal endophyte culture supernatants (FECS) on the growth of *T. cuspidata* cells. FECS (4, 6 and 8 g) was added to suspension cultures at day 10 of cultivation. Data are given as mean ± S.D. DW = dry weight.