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Short communication

Interactions of Taxol-producing endophytic fungus with its host (*Taxus* spp.) during Taxol accumulation

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Abstract

Endophytic fungi (*Fusarium mairei*) culture broth (EFCB) was added to cell suspension cultures of *Taxus cuspidata*. After 5 days, cultures of *T. cuspidata* given 4 ml of EFCB produced a maximal yield of 6.11 mg/l paclitaxel, with a release ratio of 75%, 2- and 6.8-fold, respectively, greater than the controls. The active element in EFCB is an exopolysaccharide of ~79 kD. Endophytic fungi produced 0.19 mg/l of paclitaxel in its producing medium. However, when the supernatant of *Taxus* cell suspension cultures from day 20 was added to the paclitaxel-producing medium, the biomass of fungi decreased by 24% and the yield of paclitaxel by 45%. In a co-culture system of plant and fungus, the yield of paclitaxel (12.8 mg/l) was >2-fold higher than that in the EFCB-treatment system.

Keywords: Co-cultivation; Endophytic fungi; Paclitaxel; Plant cell suspension culture; *Taxus*

1. Introduction

Paclitaxel is a potent antimitotic agent with excellent activity against a range of cancers (Suffness and Wall, 1995). Several methods have been developed for paclitaxel production, e.g. total chemical synthesis (Holton et al., 1995a; Nicolaou et al., 1994), semisynthesis from its precursor (Commercn et al., 1995; Holton et al., 1995b) and plant cell culture, etc. Plant cell culture has been an attractive alternative (Gibson et al., 1993). Additionally, certain endophytic fungi associated with *Taxus* produce paclitaxel, although the amounts are very small compared to those produced by various *Taxus* species (Stierle et al., 1993; Strobel et al., 1996; Wang et al., 2000). Thus, new and promising methods to produce paclitaxel can be envisioned.

We derived a paclitaxel-producing endophytic fungus from the inner bark of the China maire yew and identified it as *Fusarium mairei* (Xu et al., 2006). The ecological relationships between endophytic fungi and their respective plant hosts are complex. An endophytic fungus 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. But the roles of endophytic fungi and their plant hosts during accumulation of secondary metabolites are even more complex, and the roles of *F. mairei* and its plant host (*Taxus* spp.) 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., 2005). Thus, endophytic fungi may play important roles in paclitaxel biosynthesis in the yew tree. We have developed a co-cultivation system of plant and fungus, and aim to elucidate the fungus/plant relationships during paclitaxel accumulation in the yew tree.

2. Methods and materials

2.1. Plant cell cultures

T. cuspidata (line T116) cells were grown in darkness on a gyratory shaker at 100 rpm and 25 ± 1 °C, in Gamborg's B5

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medium (Gamborg et al., 1968) supplemented with naphthaleneacetic acid (NAA) 2 mg/l, 6-benzylaminopurine (6-BA) 0.15 mg/l, casein acid hydrolysate 1 g/l and sucrose 25 g/l. An aliquot (20 ml) of a 12-day-old subcultured suspension cultures (approximately 6 g of fresh weight of cells) was inoculated into 80 ml of B5 medium in a 500 ml Erlenmeyer flask. Cell suspension cultures of *T. cuspidata* were subcultured every 12 days for three generations prior to this experiment.

2.2. Paclitaxel-producing cultures of endophytic fungus (F. mairei)

The paclitaxel-producing medium for *F. mairei* consists of glucose 80 g/l, NH_4NO_3 5 g/l, $MgSO_4$ 0.5 g/l, KH_2PO_4 0.5 g/l, $ZnSO_4$ 1 mg/l, $Cu(NO_3)_2$ 1 mg/l, $FeCl_3$ 2 mg/l and NaOAc 1 g/l (Li et al., 1998; Xu et al., 2006). An aliquot (5 ml) of 3-day-old cultures of *F. mairei* (strain K178) was inoculated into 100 ml of paclitaxel-producing medium in a 500 ml Erlenmeyer flask covered with six layers of cheesecloth. The incubation was carried out at 170 rpm and 25 °C for 12 days.

2.3. Preparation of endophytic fungi culture broth (EFCB)

F. mairei (strain K178) was cultured in a 500 ml Erlenmeyer flask containing 100 ml B5 medium on a gyratory shaker at 170 rpm and 25 ± 1 °C for 6 days. The culture broth was then filtered through four 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 the concentrated culture broth was quantified by determining the total carbohydrate via the phenol-sulfuric acid method using glucose as the standard.

2.4. Confirmation of active elements in endophytic fungi culture broth (EFCB)

Exopolysaccharide (EPS) in EFCB was obtained by precipitation with ethanol. Protein was removed from EPS with the Sevag method (Staub, 1965). The mean molecular mass of EPS was determined by high performance size-exclusion chromatography (HPSEC) system (Water 600). The chromatography was carried out by utilizing Ultrahydrogel™ Linear (300 mm × 7.8 mm id × 2) column at 45 °C, eluted with 0.1 mol/l $NaNO_3$ at 0.9 ml/min.

Esters in EFCB were extracted with ethyl acetate.

2.5. Determination of plant cell viability and biomass

The cell viability was determined by staining the cells with 2,3,5-triphenyl tetrazolium chloride (TTC) following the method of Iborra et al. (1992). The biomass was measured on a dry weight (DW) basis.

2.6. Extraction of cell-associated paclitaxel from T. cuspidata cells

For the extraction of paclitaxel 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 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 at 40 °C under vacuum. The remaining taxanes were redissolved in 1 ml of methanol and filtered through a 0.2 μm filter prior to HPLC.

2.7. Extracellular paclitaxel extraction from the culture medium of T. cuspidata cell

Extracellular paclitaxel was extracted from the culture medium using the same volume of trichloromethane three times. Quantification of the paclitaxel was performed via a reverse-phase HPLC system (Aglilent) with an XDB-C18 column (4.6 × 250 mm, 5 μm) and a mobile phase consisting of methanol:water at 65:35 (v/v) with a flow rate of 1 ml/min, and detected by a UV detector at 227 nm. Identification of paclitaxel was accomplished by comparison of retention times and LC/MS fragmentation patterns with authentic standards (Sigma).

2.8. Paclitaxel extraction from fungus cultures

Pre-frozen fungus cultures were homogenized for 10 min with a mini tissue disintegrator at 10,000 rpm and ambient temperature, and extracted three times using the same volume of trichloromethane. The taxoids obtained were determined as described for the quantification of paclitaxel from *Taxus* cultures.

2.9. Experimental protocols

To investigate the effects of endophytic fungi on paclitaxel accumulation in cell suspension cultures of *T. cuspidata*, several volumes of EFCB (2, 4 and 6 ml) were added into cell suspension cultures of *T. cuspidata* at different cultivation stages.

Additionally, to follow the effects of *T. cuspidata* cells on paclitaxel accumulation in endophytic fungi (*F. mairei*), the supernatants of *T. cuspidata* cell suspension cultures at different cultivation stages were added to the paclitaxel-producing medium of endophytic fungus. The experimental medium consists of 50% (v/v) supernatant of plant cell suspension cultures and 50% (v/v) double-concentrated paclitaxel-producing medium, and a further 5% (v/v) endophytic fungus liquid inoculums was inoculated into the medium. The cultures were grown on a gyratory shaker at 170 rpm and 25 ± 1 °C for 12 days.

A co-cultivation system of *T. cuspidata* cells with endophytic fungi was developed for studying their interactions during paclitaxel accumulation deeply. *T. cuspidata* cells were

co-cultured with *F. mairei* in a 20-l turbine stirred co-reactor (Fig. 1, china patent No. ZL200410014227.8), and the conditions established for the growth of plant cells and endophytic fungi are summarized in Table 1. *F. mairei* was inoculated into the co-reactor when *T. cuspidata* cells had grown in co-reactor for 5 days.

2.10. Statistics

The data are reported as the mean of the three independent experiments. Results are expressed as mean ± SD. The significance of differences among experimental points was determined by two sample paired *t*-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of endophytic fungi culture broth (EFCB) on the cell growth of T. cuspidata

The effects of EFCB on the growth of *T. cuspidata* cells at day 10 of cultivation are shown in Fig. 2. For 2 days after the addition of EFCB (Fig. 2A), the specific growth rates of the plant cells were 0.08, 0.04, 0.03, 0.02 day^{-1} for the control cells and the cells treated with 2, 4 and 6 ml EFCB, respectively. But mean specific growth rates of 0.15, 0.14, 0.12 and 0.12 day^{-1} were observed, respectively, during day 2–8 of

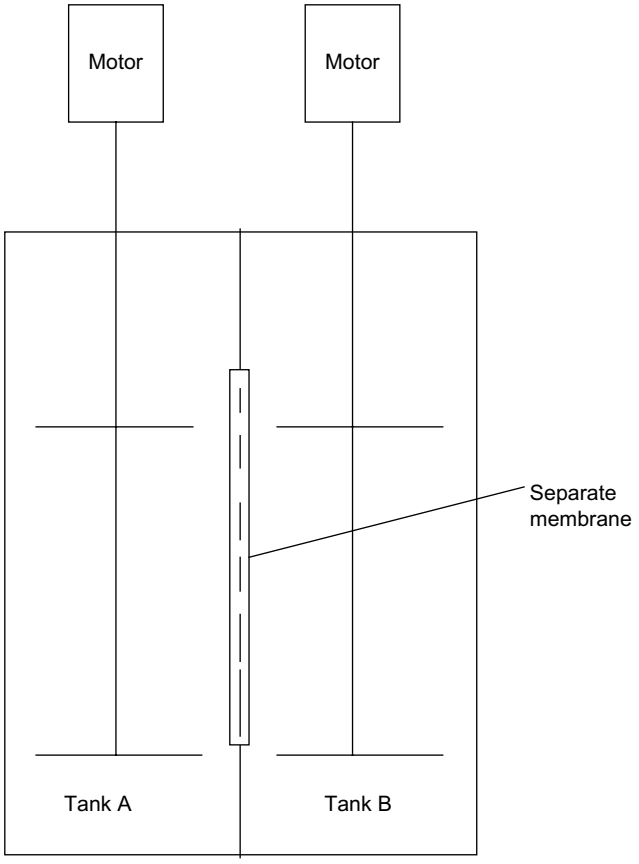

Fig. 1. Outline of the co-bioreactor structure.

Table 1
Co-cultivation parameters for plant cells and endophytic fungi in the 20-l turbine stirred co-bioreactor.

Bioreactor	Culture type	Parameters				
		Shaker (rpm)	Air flux (l/min)	Temperature (°C)	Working volume (l)	Inoculums (ml)
A (10 l)	*Taxus cuspidate*	120	3	25	6	1200
B (10 l)	*F. mairei*	180	3.5	25	6	100

Other conditions: tank pressure, 1 Kg/cm^2; separate membrane between tank A and B, 0.25 μm pyroxylin filter; working area of the center filter, 30 cm^2; medium, B5 in tanks A and B.

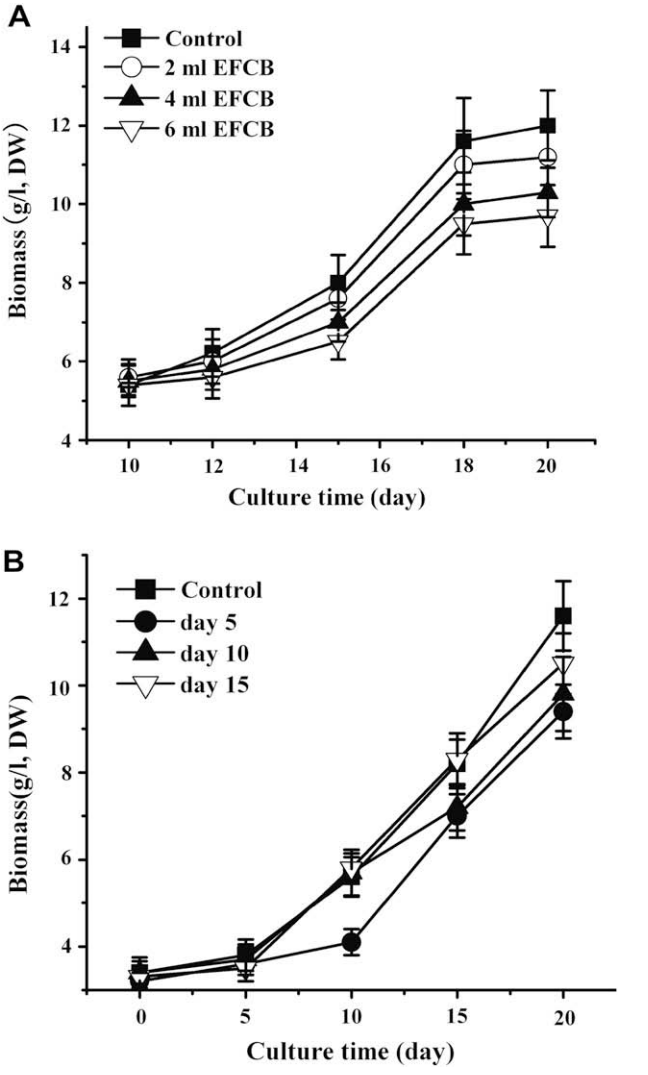

Fig. 2. Effects of the dosages of endophytic fungi culture broth (EFCB) (A) and its addition time (B) on the growth of *T. cuspidata* cells. In A, EFCB (2, 4, and 6 ml) was added to the suspension cultures at day 10 of cultivation and the samples were taken at days 10, 12, 15, 18 and 20 of cultivation. In B, EFCB (4 ml) was added to cell suspension cultures at days 5, 10 and 15 of cultivation and the samples were taken at days 0, 5, 15 and 20 of cultivation. Data represent mean values from three separate experiments ± SD. DW = dry weight.

treatment. These results indicate that the inhibition of the growth *T. cuspidata* cells by EFCB was short-lived and could be alleviated by an adaptation to EFCB. No significant differences ($P > 0.05$) were observed in *T. cuspidata* cell growth using 2 and 4 ml of EFCB-treatments compared with the control cells. But the cells treated with 6 ml of EFCB displayed a significant ($P < 0.05$) decrease in growth compared with the control cells.

Fig. 2B shows that the addition of 4 ml of EFCB at day 5, 10 and 15 of cultivation had no significant ($P > 0.05$) effects on *T. cuspidata* cell growth. From these results it may be inferred that EFCB produced no significant effects on the growth of *T. cuspidata* cell when the amount added was <4 ml.

3.2. Effects of endophytic fungi culture broth (EFCB) on the cell viability of T. cuspidata

Fig. 3 shows no significant differences ($P > 0.05$) were found in cell viability for 2 and 4 ml treatments at day 10 of cultivation compared with the control cells (Fig. 3A). But the cultures displayed a significant ($P < 0.05$) decrease in cell viability when 4 ml of EFCB were added at day 5 of cultivation and no significant ($P > 0.05$) differences when 4 ml of EFCB-treatment were added at days 10 and 15, compared with control cultures (Fig. 3B). The consumption rate of sugar in the culture medium was higher with EFCB-treatment (lower cell viability) than in the controls (with higher cell viability) (Fig. 4). It can be inferred that the EFCB-treatment enhanced the metabolism of *T. cuspidata* cells and the decrease in cell viability is due to paclitaxel accumulation rather than a direct effect of the EFCB-treatment.

3.3. Effects of endophytic fungi culture broth (EFCB) on paclitaxel accumulation and release in cell suspension cultures of T. cuspidata

Cultures treated with 4 ml of EFCB at day 5 of cultivation produced the maximal amount of paclitaxel (6.11 mg/l) and a release ratio of 75%, which were 2- and 6.8-fold higher than the values for the controls (2.98 mg/l, 11%), respectively (Table 2). The results with EFCB differ greatly from those with other elicitors used in previous studies, where the late exponential growth phase was more effective in stimulatory of paclitaxel synthesis and adding elicitors to plant cultures in the early growth phase or late stationary growth phase resulted in relatively little enhancement of paclitaxel accumulation (Wang et al., 2001).

3.4. Active elements in endophytic fungi culture broth (EFCB)

No esters were found in EFCB, although EFCB contains an EPS of mean ~79 kD (Fig. 5). The content of EPS was about 0.4% (w/v) in EFCB. EPS resulted in similar effects with EFCB on *T. cuspidata* cells.

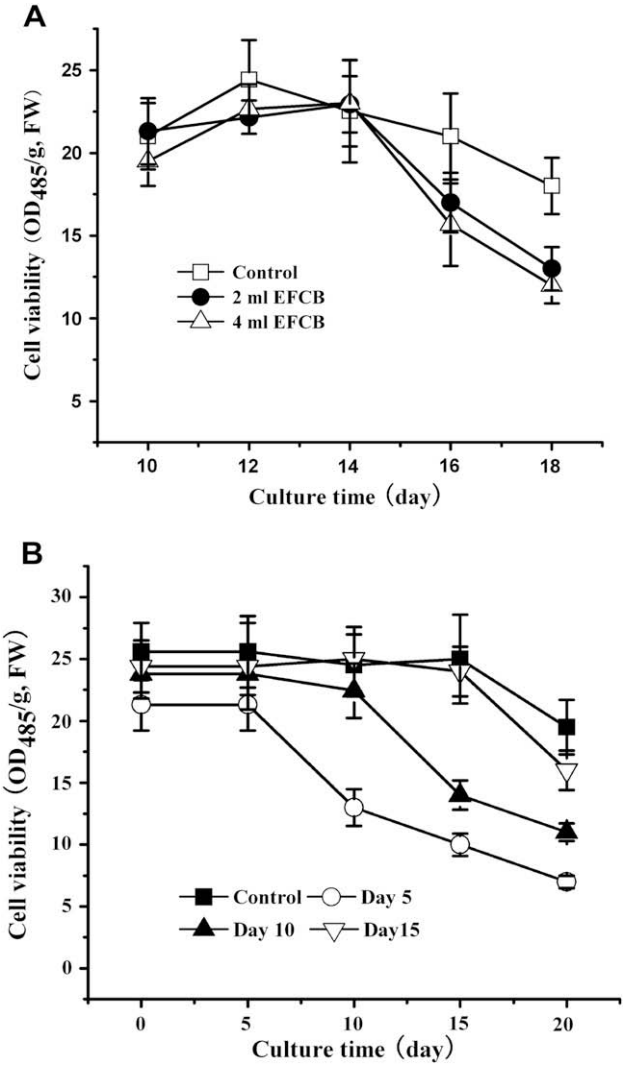

Fig. 3. Effects of the dosages of endophytic fungi culture broth (EFCB) (A) and its addition time (B) on the cell viability of *T. cuspidata*. In A, EFCB (2 and 4 ml) was added to cell suspension cultures at day 10 of cultivation and the samples were taken at days 10, 12, 14, 16 and 18 of cultivation. In B, EFCB (4 ml) was added to cell suspension cultures at days 5, 10 and 15 of cultivation and the samples were taken at day 0, 5, 10, 15 and 20 of cultivation. Data represent mean values from three separate experiments ± SD. OD = optical density, FW = fresh weight.

3.5. Effects of T. cuspidata cultures on the growth of F. mairei

Supernatants of plant cell suspension cultures of days 10 and 15 enhanced the growth of *F. mairei* (Fig. 6), but the plant cell suspension cultures of day 20 depressed the biomass of *F. mairei* by 24%, indicating that the plant cells produced substances inhibitory to *F. mairei* with the growth of plant cells.

3.6. Effects of T. cuspidata cultures on paclitaxel production in F. mairei

There are many precursors of paclitaxel in plant cell cultures (Fig. 7A), and the final contents of these precursors

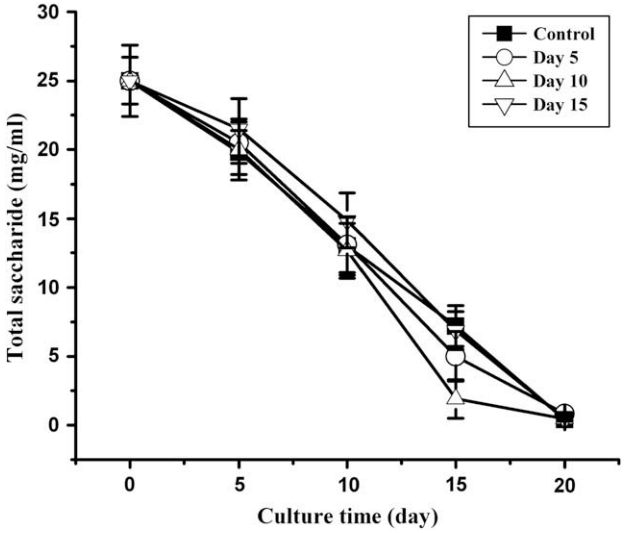

Fig. 4. Depletion curve of sugar in the culture medium treated with 4 ml endophytic fungi culture broth (EFCB) at different stages in cell suspension cultures of *T. cuspidata*. EFCB (4 ml) was added to cell suspension cultures at days 5, 10 and 15 of cultivation and the samples were taken at days 0, 5, 10 and 20 of cultivation. Data represent mean values from three separate experiments ± SD.

(10-deacetyl-baccatin III, 10-DAB; baccatin III) in fungus cultures decreased through fungi fermentation compared to the initial contents, especially the 10-DAB content, which decreased by ~50% (Fig. 7B). However, plant cell cultures did not significantly promote paclitaxel production in fungi, and the plant cell cultures of day 20 decreased the yield of paclitaxel in fungi by 45% (Fig. 7B). However, the cause of the decline in precursor content is unknown. This probably contributes to those fungi that catabolize the precursors, or the fungi that transform them to paclitaxel.

3.7. Paclitaxel accumulation in co-cultivation system of plant cells with fungi

The yield of paclitaxel in the side of plant cell cultures (Tank A) increased to 17.62 mg/l sharply after 3 days of inoculating fungi, being 24 times the yield of paclitaxel at initial day of fungus inoculation (0.72 mg/l) (Table 3). The concentration of

Table 2
Effects of endophytic fungi culture broth (EFCB) addition time on paclitaxel yield and release in suspension cultures of *T. cuspidata*.

Treatment	Paclitaxel (mg/l)			Release ratio (%)
	Extracellular	Intracellular	Total	
Untreated	0.33 ± 0.05	2.65 ± 0.27	2.98 ± 0.33	11 ± 0.6
Day 5	4.58 ± 0.43	1.53 ± 0.1	6.11 ± 0.5	75 ± 1.1
Day 10	4.09 ± 0.36	1.75 ± 0.18	5.84 ± 0.52	70 ± 1.2
Day 15	3.23 ± 0.26	1.98 ± 0.19	5.21 ± 0.44	62 ± 0.9
Day 20	0.42 ± 0.09	2.8 ± 0.25	3.22 ± 0.31	13 ± 2.3

Data represent mean values from three separate experiments ± SD. EFCB (4 ml) was added to the cell suspension cultures at days 5, 10, 15 and 20 of cultivation, and the concentrations of paclitaxel were determined at day 30. Release ratio = extracellular paclitaxel/total paclitaxel × 100%.

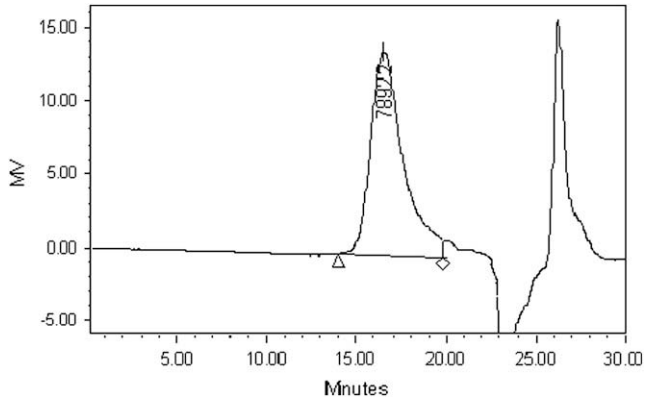

Fig. 5. Relative molecular mass of the exopolysaccharide from endophytic fungi.

paclitaxel in Tank A decreased from 17.6 mg/l at day 3 to 11.5 mg/l at day 5, this decline was likely a result that paclitaxel passed the middle separate membrane to side Tank B. Considering the paclitaxel produced in fungi cultures and the diffused paclitaxel, the mean value of paclitaxel from Tank A and B was accounted. The mean paclitaxel concentration of 12.8 mg/l at day 10 exceeded by 36-fold that at day 0 (the day of fungus inoculation). Fungi produced very low paclitaxel yield, so paclitaxel was considered to be produced mainly by plant cells. The paclitaxel content (12.8 mg/l) produced by the co-cultivation system (cultivation time of 15 days) was 2-fold higher than by EFCB-treatment (6.1 mg/l, cultivation time of 30 days), making the co-cultivation system more feasible than the EFCB-elicitation system.

The release ratio of paclitaxel in plant cell cultures increased from 15% at day 0 to 79% at day 10 (Table 3), which was similar with that in plant cell cultures treated with EFCB.

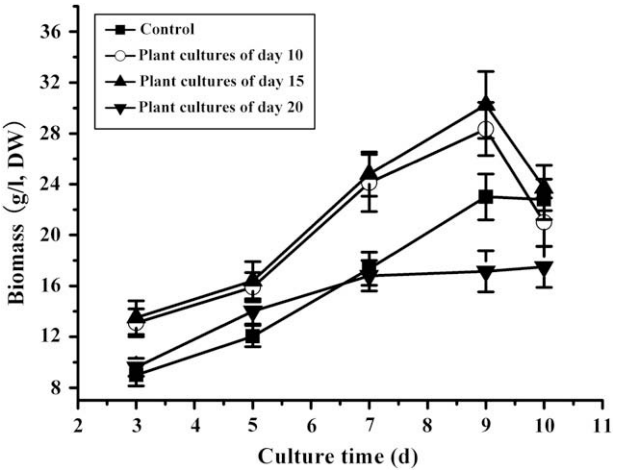

Fig. 6. Effects of plant cell cultures on the growth of endophytic fungi. The experimental medium (100 ml) consists of 50 ml supernatants of plant cell suspension cultures and 50 ml double-concentrated paclitaxel-producing medium of fungus. Data represent mean values from three separate experiments ± SD. DW = dry weight.

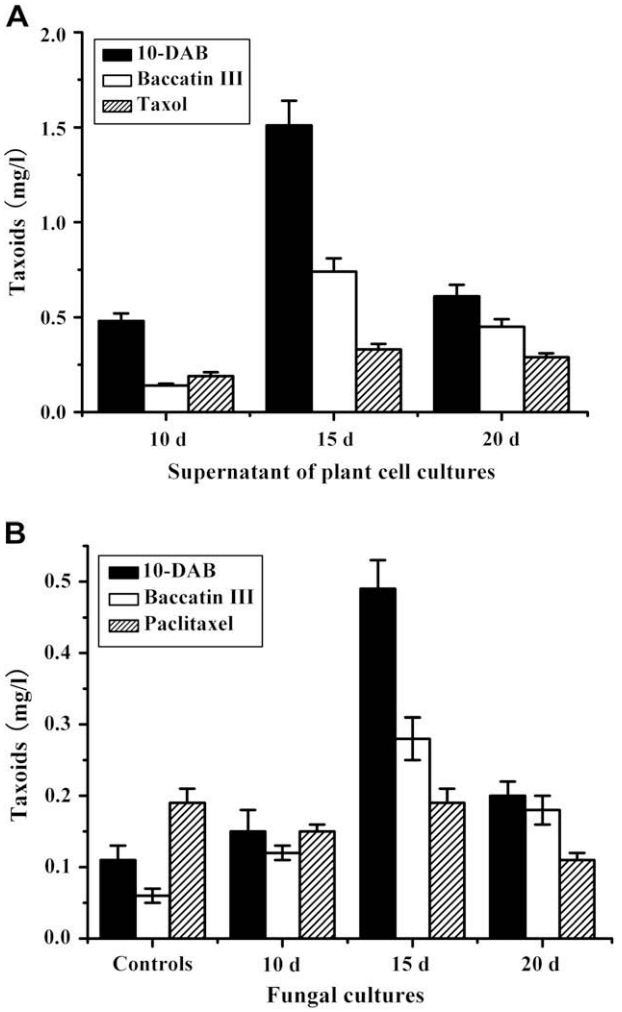

Fig. 7. Taxoids in the supernatants of plant cell suspension cultures (A) and its effects on taxoids accumulation in endophytic fungi (B). In A, 10, 15 and 20 days on horizontal axis denote the supernatants of plant cell cultures of day 10, 15 and 20, respectively. In B, 10, 15and 20 days on horizontal axis express the paclitaxel-producing cultures of fungi added with 50% (v/v) supernatants of plant cultures of days 10, 15 and 20, respectively, and the samples were taken for taxoids analysis at day 12 of fungus cultivation. Data represent mean values from three separate experiments ± SD.

Table 3
Paclitaxel accumulation in the co-cultivation system.

Bioreactor	Culture type	Paclitaxel concentrations (mg/l)					
		0 day[a]	2 days	3 days	5 days	7 days	10 days
A (10 l)	*T. cuspidate*	0.72	1.04	17.62	11.5	12.01	12.85
B (10 l)	*F. mairei*	0	0.5	0.22	7.04	12.06	12.74
Mean value from A and B		0.36	0.77	8.92	9.27	12.04	12.8
Release ratio of paclitaxel[b]		15%	28.7%	53.2%	64%	66.7%	78.8%

[a] The day of inoculating fungal inoculums after 5 days of plant cell cultivation.

[b] Release ratio of paclitaxel in plant cell cultures. Release ratio = extracellular paclitaxel/total paclitaxel × 100%. Data represent mean values from three of samples analyzed, the maximum error is less than 10%.

4. Discussion

Our results clearly show that the endophytic fungi have substantial effects on paclitaxel productivity and release in cell suspension cultures of *T. cuspidata*. EFCB has general characters of elicitor, such as activation of PAL (phenylalanine ammonium-lyase) activity, medium alkalinization, etc. (data not shown).

Previous studies showed that the diminished cell viability stressed by elicitors depressed the paclitaxel biosynthesis and contributed to unstable paclitaxel production (Yu et al., 2001, 2002a,b). The secondary metabolite production in plant cells is closely related to the cell growth and cell viability (Lindsey and Yeoman, 1983). Most elicitors (such as MJ, fungal elicitor and heavy metal ions, etc.) can cause a decrease in cell growth and cell viability (Wu and Lin, 2003). EFCB also produced the similar phenomena during the 2 days after it was added, but the growth rate of *T. cuspidata* cell stressed by EFCB can return to the normal levels of un-stressed cells after a short time (2–8 days) (Fig. 1A). It can be inferred that *T. cuspidata* cells adapt to the stress induced by EFCB, which may be a result of the development of fungus-plant symbiosis a long time ago.

With respect to the timing of EFCB addition, the previous studies (Mirjalili and Linden, 1996; Yukimune et al., 1996; Yuan et al., 2002) demonstrated the optimal timing of elicitor addition should be during the late exponential stage (days 12–15 of cultivation). However, our results (Table 2) show the highest paclitaxel yield and release was achieved when EFCB was added at day 5. There are a number of possible explanations; first, the *T. cuspidata* cells may have a tolerance against EFCB stress. Second, due to long-term development of symbiotic relationship between plant and fungus, an adaptation to EFCB stress possibly appears in the plant cells. Finally, there are few harmful components in culture medium during early culture period, so plant cells can recover from EFCB stress.

Paclitaxel is a lipophilic molecule which can therefore easily enter biological membranes (Balasubramanian and Straubinger, 1994), suggesting that at least some portion of the paclitaxel synthesized in the cells will be excreted in a passive processes. But Fornalè et al. (2002) proved that *Taxus* cells release the compound by means of a cellular ATP-dependent release mechanism. Elicitors often cause the peroxidation of *Taxus* cell membrane (Yu et al., 2001). Compared with other elicitors, *T. cuspidata* cells treated with EFCB displayed higher paclitaxel release ratio, but EFCB did not lead to the damage of the cell membrane (lower malondialdehyde and cell membrane integrity loss) and the increase in physical permeability of the cell membrane. Additionally, EFCB can accelerate the rate of sugar consumption in *Taxus* cell cultures (see Fig. 4), which suggests that EFCB probably promotes energy formation in *Taxus* cells, with the consequence enhancement of paclitaxel release, or else EFCB activates the enzymes involved in active transport of paclitaxel.

Co-cultivation of plant cells and fungi proved an efficient method of investigating their relationships and promoting

paclitaxel yield. But the control of the growth of fungi is the key to this method. The over-growth of fungi could cause an inhibition or death of plant cells as well as exhaustion of nutrients from the medium. We controlled the growth of fungi by a relatively small inoculum of fungi (1.5% v/v) and the appropriate timing of fungus inoculation. The fungi switched on the secondary metabolism of plant cells, and the secondary metabolites depressed the over-growth of fungi.

The co-cultivation system supplied a symbiosis condition for plant cell and its endophytic fungus. The exchange of available substances (e.g. elicitors produced by fungi to plant cells, inhibitors produced by plant cells to fungi, etc.) via the separate membrane in the middle of the system regulated their growths and enhanced the paclitaxel accumulation. Although the available elements in EFCB-treatment system and co-cultivation system may be similar (EPS), the stimulating effects of elicitors in EFCB-treatment system on paclitxel synthesis would be short-lived and very limited (Wang et al., 2001), and those in co-cultivation system was uninterrupted and long-lived. Thus, compared to EFCB-treatment, the co-cultivation system produced higher paclitaxel yield.

In brief, the fungi stimulated plant cells to produce and excrete paclitaxel, while the plant cells regulated the growth of fungi in their symbiotic relationship. During the long-term process of plant-fungus interactions, plant cells gradually adapt themselves to the stress of fungi. Fungi lead to few harmful effects on *Taxus* cells and improve paclitaxel production in plant cells. We believe that the co-cultivation system will be a novel and potential alternative to research plant-fungus relationships and produce paclitaxel in industry scale.

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