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Effect of Elicitors and a Biogenetic Precursor on Paclitaxel Production in Cell Suspension Cultures of *Taxus cuspidata* Var. nana

Sanro Tachinbana, Toshio Muranaka and Kazutaka Itoh
Department of Applied Bioscience, Faculty of Agriculture, Ehime University,
Tarumi 3-5-7, Matsuyama, Ehime 790-8566, Japan

Abstract: To stimulate the production of taxol (paclitaxel) by cell suspension cultures of *Taxus cuspidata* var. nana, two kinds of elicitors and a biogenetic precursor were used in F4G4 culture medium. Paclitaxel production was enhanced by each elicitor as well as by the biogenetic precursor and by a combination of the two elicitors or one elicitor and the biogenetic precursor. The amount of paclitaxel produced (16.6 mg L^{-1}) was greatest when the cell suspension cultures were conducted in F4G4 medium containing chito-heptaose (8 mg L^{-1}) and jasmonic acid (21 mg L^{-1}). The productivity was enhanced 4.1 fold compared to the control. The amount of paclitaxel produced was increased by supplying air to the cultures, though the productivity depended on the amount of air supplied. However, no enhancement of production was observed when a combination of air and chito-heptaose was provided to the cell suspension cultures.

Key words: *Taxus cuspidata* var. nana, cell suspension cultures, taxol (paclitaxel), chito-heptaose, jasmonic acid, phenylisoserine

INTRODUCTION

Taxol (Pactitaxel) was first isolated from the bark of Pacific yew, *Taxus brevifolia*, by Wani *et al.* (1971). Paclitaxel is currently the best known drug approved for use in the treatment of breast, ovarian and non-small cell lung cancer and AIDS-treated Kaposi's sarcoma (Ojima *et al*., 2002). Trees of the genus *Taxus* grow slowly, yielding relatively little paclitaxel. Also, there are restrictions on the harvesting of yew trees. Therefore, alternative sources of paclitaxel have been sought. Due to complex chemical structure, total chemical synthesis is not considered economically feasible. However, semisynthesis of paclitaxel from baccatin III isolated from the needles of *Taxus baccata* has provided an immediate and renewable source of the drug (Kingston *et al*., 1994). Alternatively, the production of paclitaxel by Taxus cell cultures has been conducted (Fett-Neto *et al.,* 1994a, b; Yukumune *et al*., 1996; Zhang *et al*., 2007). However, yields were comparatively low. Consequently, attempts have been made to enhance the productivity of Taxus cell cultures by including a biogenetic precursor (Fett-Neto *et al.* 1994a, b; Muranaka *et al*., 2004), Nutrient feeding (Choi *et al*., 2000), *in situ* extraction (Zhang *et al*., 2001), elicitor treatment with chitosan (Zhang *et al*., 2000), methyl jasmonate (Yukumune *et al*., 1996; Mirjalili and Linden, 1996; Ketchum *et al*., 1999) and jasmonic acid (Baebler *et al*., 2002) and so on.

Previously, we reported on the production of paclitaxel by oligosaccharides and an elicitor, a plant growth regulator and a biogenetic precursor in callus and cell suspension cultures of Kyaraboku, *Taxus cuspidata* var. nana, respectively (Yoshida *et al*., 2002; Muranaka *et al*., 2004). Chito-heptaose was found to be the most active elicitor among chito-oligosaccharides prepared by a partial hydrolysis of chitosan (Muranaka *et al*., 2004). Furthermore, previously, we isolated phenylisoserine methyl ester from the leaves and stems of *T. cuspidata* var. nana and suggested that it might be hydrolyzed with an enzyme (such as lipase) to phenylisoserine and incorporated into paclitaxel (Tachibana *et al*., 2005). Fleming *et al.* (1994) reported that phenylisoserine was incorporated into a side chain of paclitaxel during its biosynthesis. Jasmonic acid and its methyl ester, methyl jasmonate, have been proposed to be key signaling compounds in the process of elicitation leading to the accumulation of various secondary metabolites (Szabo *et al*., 1999). From these findings, we speculated that production of paclitaxel could be enhanced by addition of these compounds as elicitors or biogenetic precursors in cell suspension cultures of *T. cuspidata* var. nana.

This study describes the effect of two elicitors and a biogenetic precursor on the production of paclitaxel in cell suspension cultures of *Taxus cuspidata* var. nana. The effects of combining the elicitors and/or one elicitor and the biogenetic precursor are also described.

Corresponding Author: Sanro Tachinbana, Department of Applied Bioscience, Faculty of Agriculture, Ehime University, Tarumi 3-5-7, Matsuyama, Ehime 790-8566, Japan Tel: +81-89-946-9864

MATERIALS AND METHODS

Authentic taxol (paclitaxel) was purchased from Sigma Chemical Company as well as isolated from the leaves of *T. cuspidata* var. nana (Tachibana *et al*., 1994). Jasmonic acid and chito-heptaose seven hydrochloride were purchased from Sigma Chemical Company and Seikagaku Kogyo Ltd., respectively. Authentic phenylisoserine was synthesized from benzaldehyde by the method of Guo *et al.* (1993).

Plant materials: Fresh stems of *Taxus cuspidata* var. nana were collected in July 2000 from the garden of a house located in the suburbs of Matsuyama City, Ehime, Japan.

Cell suspension cultures and incubation conditions: Calluses were generated from young stems of *T. cuspidata* var. nana on Gamborg's B5 agar medium by the method described in our previous study (Yoshida *et al*., 2002). Cell suspension cultures were initiated and prepared as previously described (Muranaka *et al*., 2004).

Determination of paclitaxel production: Paclitaxel content was measured by HPLC as previously described (Murakana *et al*., 2004). Paclitaxel was confirmed by LC-Mass spectrometry with an API 2000 (positive mode) mass spectrometer. MS m/z: 854$(M+H)^+$, 569, 509, 286 (100%), 268, 240, 105. The mass spectrum of paclitaxel coincided with that of the authentic sample. Paclitaxel content was also checked by HPLC with TSKgel ODS-100S (5 μm) column (Tosoh 2×150 mm, Japan). The elution was performed by gradient condition from mixture of water containing 0.1% acetic acid (Solvent A) and acetonitrile (Solvent B) (45:55) to mixture of Solvent A and B (20:80) within 5 min and then mixture of solvent A and B (45:55) kept for 10 min. Flow rate was 0.2 mL min^{-1} and absorbance at 227 nm was measured. The experiments were repeated once with three replicates. The results shown are the average of three measurements. Statistics were carried out by Student's t-test. The deviation of each experimental value was considered significant at $p<0.05$.

Effect of elicitors on paclitaxel production: Chito-heptaose and jasmonic acid were used as elicitors. Chito-heptaose was used as free salts after the desultation of chito-heptaose seven hydrochloride. Chito-heptaose or jasmonic acid was added to the F4G4 medium after the cell suspension cultures of *T. cuspidata* var. nana (10%) had been incubated for 6 days under 120 rpm. The concentration of phenylisoserine added to the cultures was 0, 3, 5 or 8 mg L^{-1}. In the case of jasmonic acid, 0, 2.1 or 21 mg L^{-1} was added. Following the addition, incubation was continued for 15 days at 25°C under 120 rpm in the dark. After the incubation, cell extracts were obtained and the paclitaxel content was determined.

Effect of a biogenetic precursor on paclitaxel production: Phenylisoserine as a biogenetic precursor was added to the F4G4 medium after the cell suspension cultures of *T. cuspidata* var. nana (10%) had been incubated for 6 days under 120 rpm. The concentration used was 0, 0.1, 0.5, 1, or 5 mg L^{-1}. After that, the cultures were further incubated for 15 days at 25°C under 120 rpm in the dark. After a fixed incubation period, the cells were collected and taxol content was measured.

Effect of a combination of jasmonic acid and chito-heptaose on paclitaxel production: Both jasmonic acid and chito-heptaose were added to the F4G4 medium after the cultures had been incubated for 6 days under 120 rpm in the dark. The concentration of jasmonic acid used was 0, 2.1, or 21 mg L^{-1}. The concentration of chito-heptaose used was 0, 3, 5, or 8 mg L^{-1}. The cultures were then further incubated for 15 days at 25°C under 120 rpm in the dark. After a fixed incubation period, the cells were collected and paclitaxel content was measured.

Effect of a combination of phenylisoserine and chito-heptaose on paclitaxel production: Both phenylisoserine and chito-heptaose were added to the F4G4 medium after the cell suspension cultures had been incubated for 6 days under 120 rpm in the dark. The concentration of phenylisoserine used was 0, 0.1, 0.5, 1, 5, or 10 mg L^{-1}. The concentration of chito-heptaose used was 0 or 8 mg L^{-1}. The cultures were then further incubated for 15 days at 25°C under 120 rpm in the dark. After a fixed incubation period, the cells were collected and paclitaxel content was measured.

Effect of air induction and the combination of air and chito-heptaose on paclitaxel production: Air was introduced into the F4G4 medium after the cultures (10%) had been pre-incubated for 6 days under 120 rpm in the dark, while chito-heptaose was added when the incubation was started. The concentration of chito-heptaose used and amount of air supplied was 0 and 3 mg L^{-1} and 0, 1 and 3 vvm, respectively: 1 vvm means the volume of air (1 L) bubbled into 1 L of medium per hour. After that, the cultures were further incubated for 15 days at 25°C under 120 rpm in the dark. After a fixed incubation period, the cells were collected and paclitaxel content was measured.

RESULTS AND DISCUSSION

Effect of addition of chito-heptaose on paclitaxel production by cell suspension cultures of *T. cuspidata* var. nana: The production of paclitaxel was stimulated when chito-heptaose was added to the cell suspension cultures (Table 1). The production was stimulated 3 fold compared to that of the control (no addition) when the chito-heptaose (8.0 mg L^{-1}) was added to the culture medium and the cultures incubated for 15 days at 25°C in the dark. The results show that chito-heptaose, a kind of oligosaccharide, stimulate the production of paclitaxel in cell suspension cultures of *T. cuspidata* var. nana.

The stimulation of paclitaxel production by oligosaccharides in cell suspension cultures of *Taxus* sp. has been reported by Wang *et al.* (2001), Yuan *et al.* (2001), Muranaka *et al.* (2004) and Zhang *et al.* (2007). Wang *et al.* (2001) reported that the amount of paclitaxel produced was increased about two-fold compared to the control when fungal oligosaccharides prepared from *Aspergillus niger* were added to cell suspension cultures of *T. chinensis*. They also reported that the level of production was increased seven-fold compared to the control by repeated elicitation with the oligosaccharides and repeated renewal of the medium. Yuan *et al.* (2001) reported that fungal oligosaccharides prepared from *Fusarium oxysprum* stimulated paclitaxel production to a level about seven times higher than that of the control when added to cell suspension cultures of *T. chinensis* var. mairei. Zhang *et al.* (2007) reported that productivity was enhanced 2 fold by chitosan-adapted cells in cell suspension cultures of *T. chinensis,* on the yield of paclitaxel increased by 20- and 12.1-fold in chitosan-adapted cells elicited by methyl jasmonate. In contrast, Linden *et al.* (2000) reported that oligosaccharides derived from chitin and chitosan did not stimulate paclitaxel production in cell suspension cultures of *T. canadensis*, however, production was stimulated by the addition of oligosaccharides and methyl jasmonate and the oligosaccharides potentiated the methyl jasmonate-induced production of paclitaxel.

From the results mentioned above, we found that chito-heptaose, a kind of oligosaccharide, acts as an elicitor in cell suspension cultures of *T. cuspidata* var. nana and the degree of elicitation with chito-heptaose was similar to that reported by Wang *et al.* (2001).

Effect of a biogenetic precursor, phenylisoserine, on paclitaxel production by cell suspension cultures of *T. cuspidata* var. nana: To stimulate the production of paclitaxel, phenylisoserine was added to the cell suspension cultures of *T. cuspidata* var. nana. The level of production was enhanced 3.4 fold compared to that of the control (no addition) when the phenylisoserine (0.5 mg L^{-1}) was added and the cultures were incubated for 15 days at 25°C under 120 rpm in the dark (Table 1). The results show that phenylisoserine stimulated the production of paclitaxel in cell suspension cultures of *T. cuspidata* var. nana. However, production decreased when the amount of phenylisoserine added exceeded 0.5 mg L^{-1} (Table 1).

Table 1: Amount of paclitaxel produced on addition of chito-heptaose and/or phenylisoserine to cell suspension cultures of *Taxus cuspidata* var. nana

		Phenylisoserine (mg L^{-1})				
Concentration		0	0.1	0.5	1	5
Chito-heptaose	0	0.024*1	0.019*1	0.082*1	0.045*1	0.042*1
(mg L^{-1})	8	0.073	0.041	0.091	0.063	0.059

*1: Paclitaxel content (%, of dried cells)

Several reports on the stimulation of paclitaxel production by biogenetic precursors such as phenylalanine and benzoic acid have been published. Fett-Neto *et al.* (1994a) found that production was stimulated about 4.5 and 4 fold compared to the control when phenylalanine (8.25 mg L^{-1}) and benzoic acid (6.1 mg L^{-1}) were added to cell suspension cultures of *T. cuspidata* and decreased when the amount added exceeded 8.25 and 6.1 mg L^{-1}, respectively. Muranaka *et al.* (2004) reported that paclitaxel production was enhanced 3.9 fold compared to the control when phenylalanine (8.25 mg L^{-1}) was added to cell suspension cultures of *T. cuspidata* var. nana. and the cultures were incubated for 30 days at 25°C under 120 rpm in the dark. In addition, Furmanowa *et al.* (2000) reported that paclitaxel production was enhanced about 4.6 fold compared to the control when 20 mg L^{-1} of phenylalanine was added to callus cultures of *T. cuspidata.*

There has been no report on the stimulation of paclitaxel production by the addition of phenylisoserine to cell suspension cultures of *Taxus* sp. This is the first report that phenylisoserine enhanced the production of paclitaxel in cell suspension cultures of *Taxus* sp.

Effect of jasmonic acid on paclitaxel production by cell suspension cultures of *T. cuspidata* var. nana: The level of paclitaxel production was highest when 21 mg L^{-1} of jasmonic acid was added to the cell suspension cultures. The productivity of the cell cultures was enhanced 3.5 fold compared to the control (Table 2). There is no report on the stimulation of paclitaxel production by jasmonic acid in cell suspension cultures of *T. cuspidata* var. nana.

Baebler *et al.* (2002) reported that the production of paclitaxel was stimulated 19 fold and 4 fold in the cells and medium, respectively, compared to the control when

Table 2: Amount of paclitaxel produced on addition of jasmonic acid and/or chito-heptaose to cell suspension cultures of *Taxus cuspidata* var. nana

		Jasmonic acid (mg L^{-1})		
Concentration		0	2.1	21
Chito-heptaose	0	0.024*1	0.071*1	0.083*1
(mg L^{-1})	3	0.042	0.046	0.064
	5	0.037	NC*2	NC*2
	8	0.073	0.067	0.098

*1: Paclitaxel content (%, of dried cells); *2: NC: not conducted

jasmonic acid (21 mg L^{-1}) was added to cell suspension cultures of *T. media* and incubated for 13 days in B5 liquid medium. They also reported that jasmonic acid as well as methyl jasmonate is an effective elicitor of taxane production in cell suspension cultures of *Taxus* sp. Methyl jasmonate strongly promotes the biosynthesis of taxanes including paclitaxel in plant cell cultures (Mirjalili and Linden, 1996; Yukumune *et al*., 1996; Ketchum *et al*., 1999; Wang *et al*., 2004; Kim *et al*., 2005; Zhang *et al*., 2007). Yukumune *et al.* (1996) reported that the production of paclitaxel was stimulated about 4 and 40 fold compared to the control when 22.4 mg L^{-1} of methyl jasmonate was added to cell suspension cultures of *T. media* and *T. baccata* and incubated for 14 days in WPM liquid medium, respectively.

From the results mentioned above, we found that jasmonic acid acts as an elicitor in cell suspension cultures of *T. cuspidata* var. nana, though it was not as effective as methyl jasmonate.

Effect of the combination of chito-heptaose and phenylisoserine on paclitaxel production by cell suspension cultures of *T. cuspidata* var. nana: The level of paclitaxel production was enhanced 3.8 fold compared to the control when both phenylisoserine (0.5 mg L^{-1}) and chito-heptaose (8.0 mg L^{-1}) were added to the cell suspension cultures of *T. cuspidata* var. nana (Table 1). However, production decreased when the amount of phenylisoserine and chito-heptaose added exceeded 0.5 and 8.0 mg L^{-1}, respectively. The productivity on addition of chito-heptaose and phenylisoserine was enhanced 3 and 3.4 fold compared to the control (Table 1). These results showed a synergistic effect of phenylisoserine and chito-heptaose on paclitaxel production.

Zhang *et al.* (2000) reported that paclitaxel production was enhanced by adding a combination of elicitors (biotic and abiotic elicitors) to cell suspension cultures of *Taxus chinensis*. Productivity was stimulated a maximum of about 40 fold compared to the control when chitosan (50 mg L^{-1}), methyl jasmonate (60 μM) and Ag+ (30 μM) were added and the cultures were incubated for 10 days in B5 liquid medium. From the results obtained here and by Zhang *et al.* (2000), it was suggested that the production of paclitaxel could be enhanced by adding a combination of elicitors or an elicitor and a biogenetic precursor to cell suspension cultures of *Taxus* sp.

Effect of the combination of chito-heptaose and jasmonic acid on paclitaxel production by cell suspension cultures of *T. cuspidata* var. nana: The level of paclitaxel production was enhanced 4.1 fold compared to the control when both jasmonic acid (21 mg L^{-1}) and chito-heptaose (8.0 mg L^{-1}) were added to the cell suspension cultures of *T. cuspidata* var. nana (Table 2). However, production decreased when the amount of jasmonic acid and chito-heptaose added in combination was less than 21 and 8.0 mg L^{-1}, respectively. Linden and Phisalaphong (2000) reported that oligosaccharides derived from chitin and chitosan did not stimulate paclitaxel production, however, the oligosaccharides potentiated methyl jasmonate-induced production of paclitaxel in cell suspension cultures of *T. canadensis*. The results obtained here showed not the same but a similar tendency to those obtained by Linden and Phisalaphong (2000). Khosroushahi *et al.* (2006) reported that paclitaxel production was improved about 16 fold compared to the control by a combination of two different concentrations of several elicitors such as methyl jasmonate, salicylic acid and fungal elicitor in 2-stage suspension cultures of *T. baccata*.

The results obtained here showed that a combination of elicitors and/or an elicitor and a biogenetic precursor could be useful for enhancing the production of paclitaxel in cell suspension cultures of *Taxus cuspidata* var. nana. The maximum production of paclitaxel with a combination of chito-heptaose and jasmonic acid was 16.6 mg L^{-1}. Kim *et al.* (2005) reported that paclitaxel production on elicitation with methyl jasmonate in cell suspension cultures of *T. cuspidata* was about 40 mg L^{-1}. The value obtained here was therefore lower than that reported by Kim *et al.* (2005). Kyaraboku, *T. cuspidata* var. nana, a dwarf variety of ichii, *T. cuspidata* may be used as a new cultivar for production of paclitaxel by cell cultures of *Taxus* sp.

Effect of air supply on paclitaxel production by cell suspension cultures of *T. cupidata* var. nana: The level of paclitaxel production was enhanced 1.7 fold compared to the control when air (1 vvm) was bubbled into the cell suspension cultures of *T. cuspidata* var. nana (Table 3). However, the production decreased when the amount of air exceeded 1.0 vvm. As shown in Table 3, the cells grew according to the amount of air introduced into the cultures. The cells increased in number 2.7 fold when

Table 3: Effect of air and chito-heptaose on paclitaxel production in cell suspension cultures of *Taxus cuspidata* var. nana

Condition	Cell growth (%, of control)	Paclitaxel content (%, of dried cells)
Control (no air induction)	100	0.023
Air induction (0.5 vvm)	195	0.018
Air induction (1 vvm)	216	0.039
Air induction (3 vvm)	272	0.027
Air induction(1 vvm) + Chito-heptaose*1	270	0.019

*1: Chito-heptaose (3 mg L^{-1}) was added to the cell suspension cultures

3.0 vvm of air was bubbled into the cultures. One of the most important factors for the growth rate of cells in plant cell cultures is the supply of air (Tanaka, 1992). However, the amount of paclitaxel produced did not increase with the increase in the amount of air introduced. Navia-Osorio *et al*. (2002) reported that paclitaxel and baccatin III production was increased about 10 fold compared to that in the cell suspension in a batch mode when cell suspension cultures of *T. baccata* and *T. wallichiana* were conducted for 28 days in an airlift bioreactor. They pointed out two reasons for the difference: the capacity of taxane synthesis and the difference in the increase in biomass (cell growth). Therefore, air induction may not directly affect production of paclitaxel, though it affects the growth of cells.

Effect of the combination of air induction and chito-heptaose on paclitaxel production: The level of paclitaxel production was enhanced 1.7 fold when the cell suspension cultures of *T. cuspidata* var. nana were supplied with air (1 vvm), as shown in Table 3. However, the production decreased about 0.8 fold compared to the control (no air induction) when the cultures received both air and chito-heptaose. The growth of cells increased about 2.7 fold compared to the control (no air induction) when a combination of air induction and chito-heptaose were added and the cells were cultured for 15 days at 25°C under 120 rpm in the dark. We found here and previously that paclitaxel production was stimulated by the addition of chito-heptaose to cell suspension cultures of *T. cuspidata* var. nana. However, when both chito-heptaose and air were supplied to cell suspension cultures, the amount of paclitaxel produced decreased compared to the control. The reason for this decrease is unclear. It should be noted that a combination of chito-heptaose and air induction may decrease the production of paclitaxel in cell suspension cultures of *Taxus* sp.

CONCLUSION

The production of paclitaxel was stimulated from 3 to 4.1 fold compared to the control by the addition of an elicitor (chito-heptaose or jasmonic acid) or a biogenetic precursor (phenylisoserine), by a combination of the two elicitors and by a combination of one elicitor and the biogenetic precursor, in cell suspension cultures of Kyaraboku (*T. cuspidata* var. nana). Maximum production of paclitaxel (16.6 mg L^{-1}) was obtained when cell suspension cultures of *Taxus cuspidata* var. nana having received jasmonic acid and chito-heptaose were cultured for 15 days at 25°C in the dark. The amount of paclitaxel produced was increased by supplying air to the cell suspension cultures, though productivity depended on the amount of air supplied. However, no enhancement of paclitaxel production by a combination of air induction and chito-heptaose was recognized. From the results obtained here, Kyaraboku, *T. cuspidata* var. nana, may be used as a new cultivar for production of paclitaxel by cell cultures of *Taxus* sp.

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