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Effect of Culture Medium, Elicitors, A Plant Growth Regulator and a Biogenetic Precursor on Taxol Production in Cell Suspension Cultures of *Taxus cuspidata* Variety Nana

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Abstract: To find a suitable culture medium for both cell growth and taxol production, cell suspension cultures of *Taxus cuspidata* var. nana were conducted using of six kinds of culture medium (B5C2, S2FG, SH, S8, F4 and F4G4). Taxol was produced in all media. However, the amount of production of taxol was largest when the cell cultures were conducted in the F4G4 medium. Furthermore, chito-oligosaccharides prepared by a partial hydrolysis of chitosan and each constituent of the chito-oligosaccharides (from glucosamine to chito-heptaose) as elicitors, gibberellin A3 (GA3) as a plant growth regulator and phenylalanine as a biogenetic precursor were added to the cell suspension cultures to enhance taxol production. It was found that the amount of production of taxol was increased 7.0 fold over the control by addition of chito-heptaose. However, little if any effect of GA3 and phenylalanine on taxol production was recognized.

Key words: *Taxus cuspidata* var. nana, cell suspension cultures, taxol, chito-oligosaccharides, chito-heptaose

INTRODUCTION

In the 1960's, the National Cancer Institute, U.S.A. conducted a screening of higher plants for potential anti cancer drugs^[1]. The Institute found that taxol in the bark of Pacific yew, *Taxus brevifolia*, has strong anti carcinogenic activity^[2]. Taxol activates the aggregation of microtubules in the G2/M-phase of the cell division cycle and promotes the polymerization of tubulin^[3]. Taxol has a powerful effect on ovarian cancer, breast cancer, lung cancer and so on via the inhibition of cell growth^[4]. However, as *Taxus brevifolia*, grows slowly and yields relatively small amounts of taxol, attempts have been made to produce taxol by chemical synthesis^[5-7] semisynthesis^[8] plant tissue culture^[9-20] and fermentation^[21,22]. Chemical synthesis requires multiple (over 20) steps and the total yield is very low. The most promising methods for industrial production seem to be semisynthesis and plant tissue culture. However, in order to produce taxol in plant tissue cultures, problems, such as how to increase productivity have to be solved.

Previously, study reported on the production of taxol by callus cultures of Kyaraboku, *Taxus cuspidata* var. nana^[20]. This paper describes the effect of the medium on the production of taxol in cell suspension cultures of Kyaraboku. Furthermore, the effect of enhancement of taxol production by addition of elicitors, a plant growth regulator and a biogenetic precursor to the culture medium is described.

MATERIALS AND METHODS

This research project was carried out in the Faculty of Agriculture, Ehime University, Japan during 1999-2002 in an effort to produce taxol from cell suspension cultures of *Taxus cuspidata* var. nana. Authentic taxol was purchased from Sigma Chemical Company as well as isolated from the leaves of *T. cuspidata* var. nana^[23]. Chitosan and, Gibberellin-A3 (GA3) and phenylalanine were purchased from Seikagaku Kogyo Ltd. and Wako Pure Chemical Industries, Ltd., respectively. Authentic chito-oligosaccharides (glucosamine to chitoheptaose as each respective hydrochloride) were obtained from Wako Pure Chemical Industries, Ltd.

Plant materials: Fresh stems of *Taxus cuspidata* var. nana were collected in July 1999 from the garden of a house located in the suburbs of Matsuyama City, Ehime, Japan.

Cell suspension cultures and incubation conditions: Calluses were induced from young stems of *T. cuspidata* var. nana on Gamborg's B5^[24] by the method described in our previous paper^[20]. To find a suitable medium for both cell growth and taxol production, six media (B5C2, S2FG, SH, S8, F4 and F4G4, Table 1) were used. [B5C2 medium (B5 medium supplemented with 2×B5 vitamins, 30 g l⁻¹ saccharose, 4 mg l⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid), 1 mg l⁻¹ kinetin and 0.2 g l⁻¹ casamino acids), S2FG

medium (B5 medium supplemented with 2×B5 vitamins, 20 g l⁻¹ saccharose, 25 g l⁻¹ glucose, 25 g l⁻¹ fructose, 5 mg l⁻¹ NAA (1-naphthaleneacetic acid), 0.2 mg l⁻¹ kinetin and 0.2 g l⁻¹ casamino acids), SH^[29] medium, S8 medium (B5 medium supplemented with 2×B5 vitamins, 80 g l⁻¹ saccharose, 5 mg l⁻¹ NAA, 0.2 mg l⁻¹ kinetin and 0.2 g l⁻¹ casamino acids), F4 medium (B5 medium supplemented with 2×B5 vitamins, 40 g l⁻¹ fructose, 5 mg l⁻¹ NAA, 0.2 mg l⁻¹ kinetin and 0.2 g l⁻¹ casamino acids), F4G4 medium (B5 medium supplemented with 2×B5 vitamins, 40 g l⁻¹ glucose, 40 g l⁻¹ fructose, 5 mg l⁻¹ NAA and 0.2 g l⁻¹ casamino acids)]

To obtain fine and uniform cells, pre-incubation was carried out using each medium. Fresh callus (about 10 g) cultivated for 8 weeks after one subculture on B5 medium was placed in an Erlenmeyer flask and added to each medium to make a cell concentration of 10%. Pre-incubation was conducted for one week at 25°C with a rotary shaker at 120 rpm in the dark. After the pre-incubation, each cell suspension culture was filtered with a büchner funnel containing sterilized filter paper. Fresh cells (about 5 g) were taken in an Erlenmeyer flask and added to each liquid medium to make a 10% cell concentration. The suspension cultures were conducted under the same conditions described above.

Determination of cell growth and taxol production: Cell growth was measured using PCV (Packed cell volume). A 2 ml aliquot of the culture was taken every 3 days, poured into a centrifugal tube with 6 ml of fresh medium and centrifuged for 5 min under 1500 rpm. PCV was measured by recording the volume of cells as a percentage of total culture volume. The measurement was continued for 21 days. Furthermore, to conduct continuous cell suspension cultures, PCV was measured every 3 days for 63 days in the manner described above: i.e. the cells were cultured for 21 days, transferred to fresh medium and cultured for another 21 days, again transferred to fresh medium and cultured a final 21 days.

Taxol content was measured in the cells after incubation for 30 days under the conditions described above. The cells obtained by filtration of the cultures were freeze-dried for one week, ground with a pestle in a mortar and extracted with 50% methanol for one week at room temperature. The extraction was repeated and the solution was concentrated under reduced pressure to obtain extracts. The extracts were separated with dichloromethane and water (1:1) by the method of Witherup *et al.*^[25] and gave dichloromethane solubles. The amount of taxol in the solubles was determined by HPLC performed on a reverse phase column (SUPBLCOSIL TM-LC-F) in a Shimadzu LC 10A liquid chromatograph equipped with a UV (ultraviolet) detector

(wavelength: 227 nm) by isocratic elution with acetonitrile-tetrahydrofuran (THF)-water (17 : 28 : 55 (v/v)) as the mobile phase. The flow rate was 1.5 ml min⁻¹ and all chromatograms were plotted at the absorbance maximum of taxol, 227 nm. The experiment was independently repeated three times and the results shown are the average of three measurements, the deviation of each experimental value being within 12%.

Effect of elicitors on taxol production:

Chito-oligosaccharides and each constituent of the chito-oligosaccharides (from glucosamine to chito-heptaose) were used as elicitors. Chito-oligosaccharides were prepared by a partial hydrolysis of chitosan according to the method of Kikkawa *et al.*^[26]. Glucosamine, chito-biose, chito-triose, chito-tetraose, chito-pentaose, chito-hexaose and chito-heptaose were used as free salts after the desulfation of each chito-oligosaccharide purchased from Wako Pure Chemical Industries, Ltd. with the respective hydrochloride.

Chito-oligosaccharides were added to the F4G4 medium after the cell suspension cultures of *T. cuspidata* var. nana had been incubation for 6 days. The concentration used was 1.0, 5.0 and 10.0 mg l⁻¹. Following the addition, incubation was continued for 30 days at 25°C in the dark. After the incubation, cell extracts were obtained and the taxol content was determined as described above. Each of the seven kinds of chito-oligosaccharide was added to the F4G4 medium of a concentration of 5 mg l⁻¹, incubation was continued for 30 days at 25°C in the dark and the taxol content of the cells was measured in the same way described above.

Effect of a plant hormone on taxol production:

GA3 as a plant growth regulator was added to the F4G4 medium after the cell suspension cultures of *T. cuspidata* var. nana had been incubated for 6 days. The concentration used was 1.0, 5.0 and 10.0 mg l⁻¹. GA3 as a 50% acetone solution was added to the medium and the cells were cultured for 30 days at 25°C in the dark. After a fixed incubation period, the cells were collected and taxol content was measured as described above.

Effect of a biogenetic precursor on taxol production:

Phenylalanine as a biogenetic precursor was added to the F4G4 medium after the cell suspension cultures of *T. cuspidata* var. nana had been incubated for 6 days. The concentration used was 1.65, 8.25 and 16.50 mg l⁻¹. After that, the cultures were further incubated for 30 days at 25°C in the dark. After a fixed incubation period, the cells were collected and taxol content was measured as described above.

RESULTS AND DISCUSSION

Cell growth and taxol production by cell suspension

cultures: Six kinds of culture media were used for cell suspension cultures of *T. cuspidata* var. *nana* (Table 1). The cells grew in all media, however, a clear difference in the growth rate of cells in each medium was not observed. The cells grew relatively well in S2FG, F4 and F4G4. Numbers increased 1.30 fold at maximum when the cultures were carried out for 15 days in the F4G4 medium. A similar growth rate was observed in S2FG and F4. It was considered that the logarithmic growth phase of cells was from 6 to 13 days of the incubation, the stationary phase was from 13 to 20 days and the death phase was after 21 days (Fig. 1).

The taxol content of the cells incubated in the B5C2, S2FG, SH, S8 and F4 media was about 0.01 to 0.02% of dried cells. The taxol content of the cells incubated in the F4G4 medium was greatest, at 0.05% of dried cells (Fig. 2). From the results obtained here, it was found that F4G4 was the best medium for taxol production as well as cell growth in the cell suspension cultures of *T. cuspidate* var. *nana*. Therefore, the F4G4 medium was used in subsequent experiments.

It is said that saccharose is a preferable carbon source for growth of cells in plant cell cultures. But saccharose is rapidly metabolized to glucose and fructose

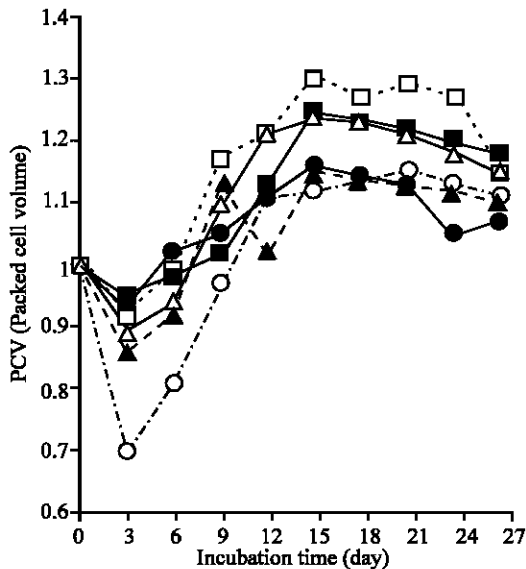


Fig. 1: Effects of media used for cell suspension cultures of *T. cuspidata* var. *nana*

Notes ●: B5C2; ■: S2FG; ▲: SH; ○: S8; △: F4; □: F4G4. For details on each medium, refer to Table 1

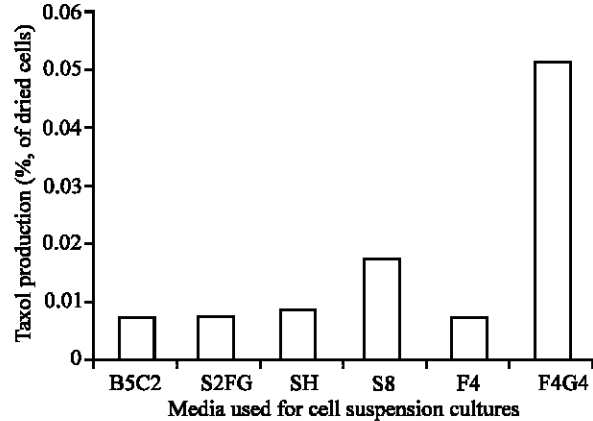


Fig. 2: Effects of medium on taxol production in cell suspension cultures of *T. cuspidata* var. *nana*

Note: For details on each medium, refer to Table 1

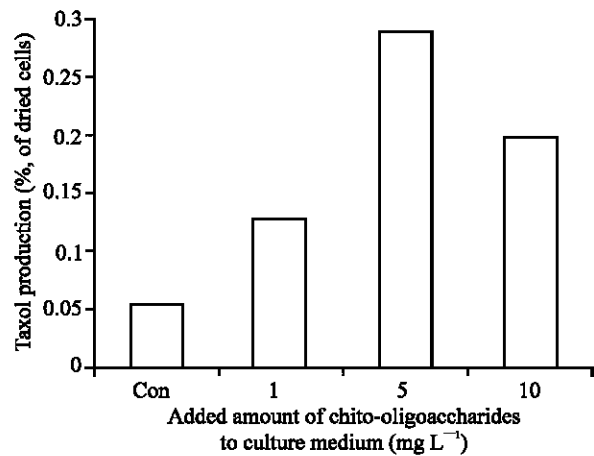


Fig. 3: Effect of chito-oligosaccharides on taxol production in cell suspension cultures of *T. cuspidata* var. *nana*

Note: Chito-oligosaccharides were added to cell suspension cultures 6 days after the incubation was started. The cultures were further incubated for 30 days after the addition. Con: Control

in cultures. The use of glucose favors fast growth of cells^[11]. Kim *et al.*^[30] reported the effects of various carbohydrates on the growth of *T. brevifolia* cell cultures in growth medium. Saccharose, lactose, galactose, glucose and fructose all had a similar effect on the growth of the cell cultures. Furthermore, they reported on the effect of various 8% sugar solutions on the production of taxol. Fructose was most effective followed by galactose. Glucose was not as effective as fructose in promoting the production of taxol (around 1/3).

Table 1: Comparison of constituents in each medium used for cell suspension cultures of *T. cuspidata* var. nana

Constituents	Concentration (mg L ⁻¹)					
	B5C2	S2FG ^a	SH	S8 ^a	F4 ^a	F4G4 ^a
Growth regulator: 2,4-D	4	0	0	0	0	0
: Kinetin	1	0.2	0	0.2	0.2	0
: NAA	0	5	5	5	5	5
Polyvinylpyrrolidone	15000	0	0	0	0	0
Sugar regime						
: Saccharose	30000	20000	30000	80000	0	0
: Glucose	0	25000	0	0	0	40000
: Fructose	0	25000	0	0	40000	40000
Casamino acids	0	200	0	200	200	200
Basal medium	B5	B5	SH	B5	B5	B5

Notes: ^a: Contained a two fold higher concentration of vitamins than normal

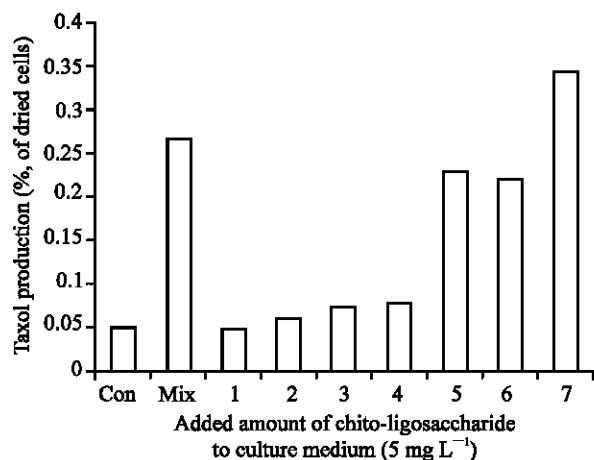


Fig. 4: Effect of chito-oligosaccharides on taxol production in cell suspension cultures of *T. cuspidata* var. nana

Note: Con: Control; Mix: Mixture of authentic sample composed of each constituent of chito-oligosaccharides.

- 1: Glucosamine; 2: Chito-biose; 3: Chito-triose;
- 4: Chito-tetraose; 5: Chito-pentaose;
- 6: Chito-hexaose; 7: Chito-heptaose

However, glucose is needed for the fast growth of cells. Therefore, it is considered that both glucose and fructose are needed for the growth of cells and production of taxol in cell suspension cultures of *T. cuspidata* var. nana.

In addition, to conduct continuous cell suspension cultures of *T. cuspidata* var. nana in F4G4 culture medium, the cultures were conducted for 21 days and repeated three times. The rate of cell growth decreased with the increase in the number of cultures (data not shown). Still, the ability to grow was maintained after three repetitions of the culture. However, it is necessary to increase the growth rate of cells by finding more suitable conditions for the stimulation of cell growth in the culture medium.

Effect of chito-oligosaccharides on taxol production: The production of taxol was stimulated significantly when

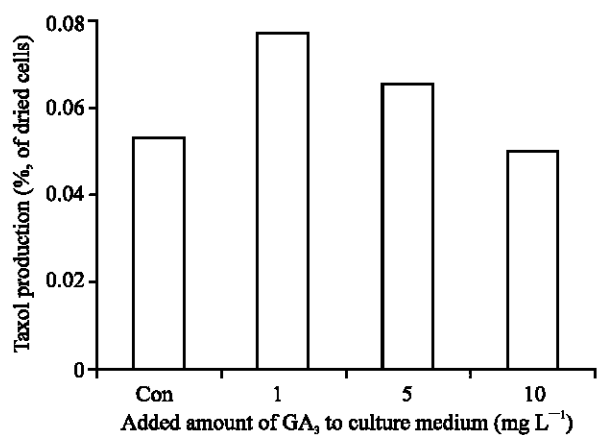


Fig. 5: Effect of gibberellin-A₃ (GA₃) on taxol production in cell suspension cultures of *T. cuspidata* var. nana

Note: GA₃ was added to the cell suspension cultures 6 days after the incubation was started. The cultures were further incubated for 30 days after the addition

chito-oligosaccharides were added to the cell suspension cultures (Fig. 3). The production was stimulated 5.3 fold compared to that of the control (no addition) when the chito-oligosaccharides (5.0 mg l⁻¹) were added to the culture medium and the cultures incubated for 30 days at 25°C in the dark. The results show that chito-oligosaccharides stimulate the production of taxol in cell suspension cultures of *T. cuspidata* var. nana. The stimulation of taxol production by oligosaccharides in cell suspension cultures of *Taxus spp.* has been reported previously. Wang *et al.*^[18] reported that the amount of taxol 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 found that the level of production was increased seven fold compared to the control by repeated elicitation with the oligosaccharides

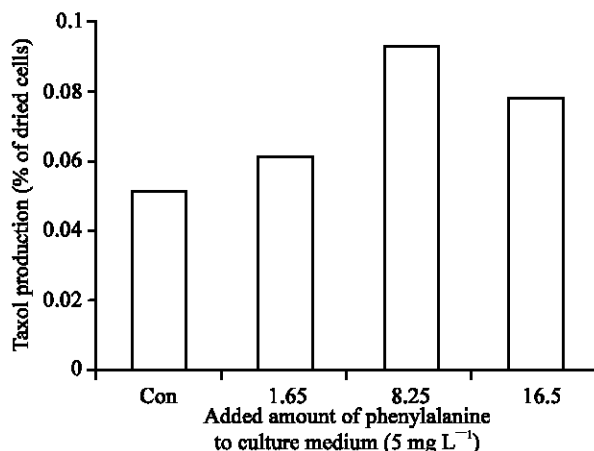


Fig. 6: Effect of phenylalanine on taxol production in cell suspension cultures of *T. cuspidata* var. nana

Note: Phenylalanine was added to the cell suspension cultures 6 days after the incubation was started. The cultures were further incubated for 30 days after the addition

and repeated medium renewal. Yuan *et al.*^[27] reported that fungal oligosaccharides prepared from *Fusarium oxysprum* stimulated taxol production to a level about seven times higher than that of the control when added to cell suspension cultures of *T. chinensis* var. mairei. Linden *et al.*^[28] found that oligosaccharides derived from chitin and chitosan did not stimulate taxol production in cell suspension cultures of *T. canadensis*. They also reported that taxol production was stimulated by addition of oligosaccharides and methyl jasmonate and the oligosaccharides potentiate methyl jasmonate-induced production of taxol. These results suggest that oligosaccharides act as an elicitor, though the degree to which they affect taxol production in cell suspension cultures of *Taxus spp.* may vary.

The active compound in chito-oligosaccharides that stimulates the production of taxol: Taxol production increased 7.0 fold compared with the control (no chito-oligosaccharide) when chito-heptaose (5 mg l⁻¹) was added to the cell suspension cultures (Fig. 4). The results show that chito-heptaose is the most active compound in chito-oligosaccharides for the stimulation of taxol production. This is the first report on the stimulation of production of taxol by chito-heptaose in cell suspension cultures of *T. cuspidata* var. nana. Yoshida *et al.*^[20] reported that chito-heptaose was the most active elicitor in chito-oligosaccharides for the production of taxol in callus cultures of *T. cuspidata* var. nana. In the present

study chito-heptaose was also the most active elicitor in chito-oligosaccharides for the production of taxol in cell suspension cultures of *T. cuspidata* var. nana.

Linden *et al.*^[28] reported that taxol production was not stimulated by N-acetylchito-hexaose alone, but by N-acetylchito-hexaose and methyl jasmonate together. There is no report on the stimulation of taxol production in cell suspension cultures of *Taxus spp.* with chito-heptaose. Experiment was conducted on the production of taxol in cell suspension cultures of *T. cuspidata* var. nana treated with chito-heptaose and other elicitors. The results will be published elsewhere.

Effect of a plant hormone on taxol production: The level of taxol production was highest when 1.0 mg l⁻¹ of Gibberellin A3 (GA3) was added to the cell suspension cultures. The productivity of the cell cultures was enhanced 1.5 fold compared to the control. However, taxol production decreased when the amount of GA3 added was over 1.0 mg l⁻¹ (Fig. 5). From the results obtained here, a growth regulator such as a plant growth regulator did not affect the stimulation of production of secondary metabolites. Fett-Neto *et al.*^[10] reported that GA3 stimulate callus growth without affecting taxol production. The results obtained here support those obtained by Fett-Neto *et al.*^[10].

Effect of a biogenetic precursor on taxol production: The level of taxol production was enhanced 1.8 fold compared to the control when phenylalanine (8.25 mg l⁻¹) was added to the cell suspension cultures of *T. cuspidata* var. nana. However, production decreased when the amount of phenylalanine added exceeded 8.25 mg l⁻¹ (Fig. 6). Fett-Neto *et al.*^[11] reported that taxol production was stimulated about 4.5 fold compared to the control when phenylalanine (8.25 mg l⁻¹) was added to cell suspension cultures of *T. cuspidata* and decreased when the amount added exceeded 8.25 mg l⁻¹. These results suggest that phenylalanine stimulates taxol production, though the degree to which it affects production in cell suspension cultures of *Taxus spp.* may vary.

F4G4 was the most suitable medium for both cell growth and taxol production in cell suspension cultures of Kyaraboku (*T. cuspidata* var. nana). The level of taxol production rose 5.3 fold compared to the control when chito-oligosaccharides were added to the medium. Chito-heptaose in the chito-oligosaccharides was found to be the most active compound for the stimulation of taxol production. The production was 7.0 times higher than the control level on addition of chito-heptaose to the cell cultures. Almost no effect of gibberellin A3 and phenylalanine on taxol production was recognized.

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