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Stimulation of the Production of Taxol by Oligosaccharides in *Taxus cuspidata* Variety Nana Callus Cultures

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Abstract: Callus cultures were established on callus derived from stems of young Kyaraboku (*Taxus cuspidata* Sieb. *et* Zucc. var. nana) trees and the effect of oligosaccharides on taxol production was investigated. The production was stimulated about 5.1-fold and 1.2-fold by the addition of chito-oligosaccharides (COS) and laminaran-oligosaccharides (LOS), respectively. To find the active compound that stimulates the taxol production, COS were separated into 8 fractions (Fr.1 - Fr.8) by ion-exchange chromatography. Fr.5 was found to be the most active fraction and chitoheptaose was its most active compound. The production was stimulated 8.2-fold on addition of chitoheptaose to the calli.

Key words: *Taxus cuspidata* var. nana, callus cultures, taxol, chito-oligosaccharides, chitoheptaose, laminaran-oligosaccharides

Introduction

Kyaraboku, *Taxus cuspidata* var. nana, with dark, linear and evergreen foliage is a popular garden tree in Japan (Uehara, 1969). Kyaraboku is a dwarf variety of ichii, *Taxus cuspidata*. We have examined the extractive of *T. cuspidata* var. nana leaves and found that taxol is present, although in small amounts (Tachibana *et al*., 1994a). Taxol, a diterpene alkaloid initially isolated from the bark of *Taxus brevifolia* by Wani *et al*. (1971), has strong cytotoxic activity against breast cancer, ovarian cancer, stomach cancer and other types of cancer (Gelmon, 1994). Therefore, for taxol to be utilized as a resource of *T. cuspidata var. nana*, its production needs to be increased.

At present, the commercial source of taxol is the bark of *Taxus brevifolia*, which grows slowly and yields relatively low amounts of taxol. Taxol is also found in *Taxus* spp. plants. Many attempts have been made to produce taxol by chemical synthesis (Nicolaou *et al*., 1994; Holton *et al*., 1994ab), semi-chemical synthesis (Holton *et al*., 1995), plant tissue culture (Fett-Neto *et al*., 1992,1993,1994a, 1994b; Wickremesinhe *et al*., 1993; Tachibana *et al*., 1994b; Ketchum *et al*., 1995; Wang *et al*., 1997; Son *et al*., 2000; Zhang and Xu, 2001) and fermentation (Stierle *et al*., 1993; Strobel *et al*., 1996). However, chemical synthesis requires multiple (over 20) steps and the total yield is very low. The most-promising methods for the large-scale production of taxol seem to be semi-chemical synthesis and plant tissue culture.

Production of taxol has been conducted by the callus culture of *Taxus cuspidata* (Fett-Neto *et al*., 1992, 1993, 1994a, 1994b), *T. cuspidata* var. nana (Tachibana *et al*., 1994b), *T. cuspidata* and *T. media* (Furmanowa *et al*., 2000) and four *Taxus* spp. (Wickremesinhe *et al*., 1993). However, there have been no reports about the stimulatory effects on taxol production of treatment with oligosaccharides in callus cultures of *Taxus* spp. Therefore, we examined taxol production in *Taxus cuspidata* var. nana callus cultures and the effect of oligosaccharides on the production.

Materials and Methods

General: The research project was carried out in the Faculty of Agriculture, Ehime University, Ehime, Japan during 1999 – 2001 to produce taxol from tissue cultures of *Taxus cuspidate* var. nana. Melting point of taxol was determined on a Yanagimoto MP micro melting point apparatus and is uncorrected. FDMS data were obtained with a Jeol JMS-700 mass spectrometer. Authentic taxol was purchased from Sigma Chemical Company as well as isolated from the leaves of *T. cuspidata* var. nana (Tachibana *et al*., 1994a). Chitosan and algal laminaran were purchased from Seikagaku Kogyo Ltd. and Tokyo Kasei Kogyo Co. Ltd., respectively. Tunicase was obtained from Wako Pure Chemical Industries, Ltd. Authentic chito-oligosaccharides (glucosamine to chitoheptaose as each respective hydrochloride) and polyvinylpyrrolidone were also obtained from Wako Pure Chemical Industries, Ltd.

Plant materials, tissue culture media: Fresh stems of young garden-grown trees *T. cuspidata* var. nana were used as explant material. The stems were collected in May 1999 from the Ehime Prefectural Garden Center located in Shigenobu Town, Ehime, Japan and from the garden of a house located in the suburbs of Matsuyma City, Ehime, Japan, respectively.

Murashige–Skoog (MS) (Murashige and Skoog, 1962), Gamborg's B5 (Gamborg *et al*., 1968) and Schenk–Hildebrandt (SH) (Schenk and Hildebrandt, 1972) media were prepared. In the case of the MS and SH media, the sucrose content was 30 g/l. In the case of B5, the sucrose content was 20 g/l. The pH of all three media was adjusted to 5.6. The growth regulators added to each medium were 2,4 D (4.0 mg/L) and kinetin (0.5 mg/L). Casamino acid (1.0 g/L) and polyvinylpyrrolidone (15g/L) were also added. The media were solidified with 0.9% w/v agar.

Callus initiation and cultures: Explants were sterilized by soaking in 70% ethanol for a few minutes and by immersing in sodium hypochlorite (1% active chlorine) for 20-30 minutes with gentle stirring followed by thrice washes with sterile water. Damaged tissue was removed and segments of the stems were transferred into each of the sterilized media.

Explants were placed on surface agar solidified medium (30ml) supplemented with 2,4 D, kinetin and casamino acid in 100-ml Erlenmeyer flasks, or on 20 ml of solidified medium in 9-cm petri dishes. Petri dishes were sealed with Nescofilm. Cultures were maintained at 25° C in dark. The calli (about 1g fresh weight) that formed were transferred to 200-ml Erlenmeyer flasks each having 70 ml of B5 medium supplemented with growth regulators, casamino acid (1.0 g/L) and agar (0.9%). To find the optimum concentration of growth regulators for the proliferation of callus, combinations [(A) - (C)] of two kinds of growth regulators were added to B5 medium. The combinations were: (A): 2,4 D (12.0 mg/L) and kinetin (1.5 mg/L); (B): 2,4 D (8.0 mg/L) and kinetin (4.0 mg/L) and (C): 2,4 D (4.0 mg/L) and kinetin (0.5 mg/L). The cultures were maintained at 25° C in the dark for 30 days. All calli were kept at 25° C and were routinely subcultured onto fresh agar-solidified B5 medium supplemented with 2,4–D (4.0 mg/L) and kinetin (0.5 mg/L) every 30 days at 25° C in dark.

Fresh weights of the calli were measured directly and dry weights after freeze-drying of tissue. The growth rate was calculated from the increase in the weight of calli relative to incubation time (day).

Extraction and isolation: Fresh calli (61.7g, dry weight 8.02g) subcultured three times on the B5 medium were freeze-dried for 5 days, ground with a pestle and extracted twice with methanol and water (1:1 v/v) (100ml) for 4 days at room temperature. The resulting extracts were then concentrated under reduced pressure and partitioned between dichloromethane and water (1:1 v/v). The dichloromethane layer was dried with Na_2SO_4 and evaporated to give dichloromethane solubles (0.54g). The dichloromethane

solubles were then subjected to preparative thin layer chromatography repeatedly followed by recrystallization from methanol and water to give taxol as colourless crystals (0.8mg), m.p. 213 - 215° C. The isolated taxol was confirmed to be identical with the authentic taxol (mixed melting point and comparison of mass spectrum). FDMS m/z (rel. int.): 854 [M^+ + H] (73), 853 [M^+] (12), 836 (7), 794 (15), 568 (16), 211 (21), 210 (100), 105 (12), 61 (5), 43 (6).

Thin layer chromatography: Preparative TLC was carried out using a 1mm (preparative) layer of silica gel (Merck Kieselgel G F254). Taxol was located by UV light and by spraying with potassium dichromate in 40% sulfuric acid, then heating with a hot air dryer (McLaughlin *et al.*, 1981).

Effect of media on growth of calli and taxol production in the calli: The fresh weights of the calli induced on MS, B5 and SH media were determined every ten days for a period of 60 days. Taxol was extracted from the calli derived from *T. cuspidata* var. nana stems by the method of Witherup *et al.* (1990). Each callus was freeze-dried, pulverized and extracted with methanol and water (1:1 v/v) for one week at room temperature. The resulting extracts were evaporated *in vacuo* to give crude residues. The crude residues were subsequently partitioned between dichloromethane and water (1:1 v/v) to give, after evaporation of the solvent and drying *in vacuo*, dichoromethane solubles. The amount of taxol in a dichloromethane soluble was determined by HPLC performed on a reverse phase column (Supelcosil TM LC F) in a Shimadzu LC 10A liquid chromatograph equipped with UV (ultraviolet) detector (wave length: 227nm) by isocratic elution with acetonitrile - tetrahydrofuran - water (17: 28: 55 v/v) mobile phase. The flow rate was 1.5 ml/min, and all chromatograms were plotted at the absorbance maximum of taxol, 227nm. The dichloromethane soluble obtained was dissolved in 5 ml of methanol, and 10μl of the solution was injected into the column. The taxol in the dichloromethane soluble was identified by comparing the retention time with that of authentic taxol isolated from *T. cuspidata* var. nana (Tachibana *et al.*, 1994a) and by comparing its mass spectrum with that of the authentic taxol. A calibration curve was obtained using authentic taxol. For comparison with the content of taxol in the cultured calli, intact stems were extracted similarly and were subjected to HPLC analysis.

Preparation of oligosaccharides by a partial hydrolysis of chitosan and algal laminaran: Chito-oligosaccharides (COS) were prepared by a partial hydrolysis of chitosan according to the method of Kikkawa *et al.* (1990). Laminaran-oligosaccharides (LOS) were prepared from algal laminaran with tunicase by the method of Kobayashi *et al.* (1993).

Fractionation and analysis of chito-oligosaccharides: Chito-oligosaccharides were separated into 8 fractions (Fr.1 – Fr.8) by ion–exchange chromatography (Dowex 50W–X8H) with an aqueous hydrochloric acid and water gradient (from 1.15N HCl to 4.10N HCl). Each fraction was neutralized with ion–exchange resin (Amberlite IRA–410) and dried *in vacuo* to give a residue. Fr.5 was analyzed by HPLC performed at 30° C on a reverse–phase column (Asahipak NH_2 P–50) in a Shimadzu LC–10A liquid chromatograph equipped with a RI (refractive index) detector by isocratic elution with 100mM tetrapropylammonium hydroxide (pH 10) – acetonitrile (30: 70 v/v). The flow rate was 1.0 ml/min. Fr.5 was dissolved in 1 ml of water, and 10μl of the solution was injected into the column. The constituents (glucosamine to chitoheptaose) in Fr.5 were identified by comparing the retention time with that of authentic chito-oligosaccharides (glucosamine to chitoheptaose) and by adding authentic samples. A calibration curve was obtained using authentic chito oligosaccharides.

Treatment of calli from *T. cuspidata* var. nana with oligosaccharides: Chito-oligosaccharides and laminaran-oligosaccharides were used. One milliliter of an aqueous solution of the chito-oligosaccharides (0.5mg, 1.0mg and 5.0mg), each fraction separated by ion-exchange chromatography (1.0mg), and standard samples (glucosamine to chitoheptaose) (1.0mg) were added to the fresh calli (1g). One milliliter of an aqueous solution of the laminaran–oligosaccharides (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0mg) was added to the fresh calli (1g). After addition of the compounds, the calli were incubated for 15 days at 25° C in the dark. The amount of taxol in the calli treated with chito–oligosaccharides, each fraction separated by ion-exchange chromatography, standard samples (glucosamine to chitoheptaose) and laminaran-oligosaccharides was determined as described above. Each experiment was repeated independently three times and the results shown are the average of three measurements, the deviation of each experimental value being within 13%.

Results and Discussion

Induction and proliferation of callus from *T. cuspidata* var. nana: Callus was produced from sterile stems of *T. cuspidata* var. nana on all three media supplemented with 2,4–D (4.0 mg/L) and kinetin (0.5 mg/L) solidified with agar (0.9 %) in the dark. No callus formed in any of the media lacking growth regulators. Each callus formed within 20 days of culture on the three media. The calli generally appeared white to yellowish brown. The contamination level during the initiation in each medium was about 40 % with garden-grown plant tissues. The growth rate of each callus was greatest on SH medium followed by B5 medium (85% of that on the SH

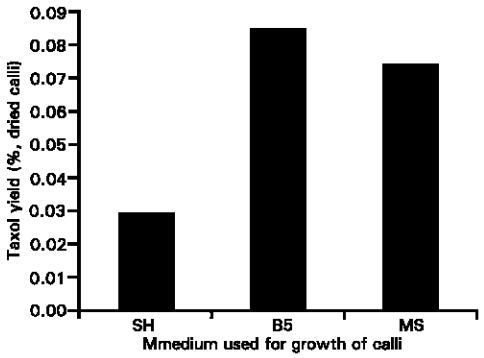

Fig. 1: Effect of medium on taxol production by calli from *T. cuspidata* var. nana
SH = Schenk and Hildebrandt medium
B5 = Gamborg's B5 medium
MS = Murashige and Shook medium

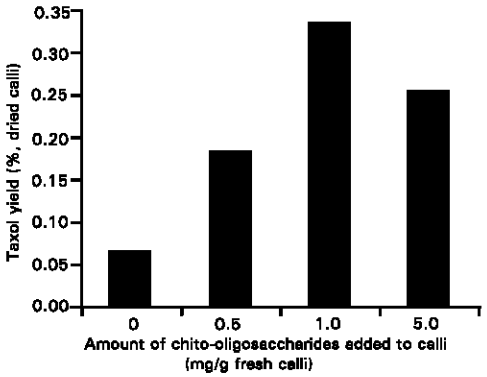

Fig. 2: Effect of chito-oligosaccharides on taxol production by calli from *T. cuspidata* var. nana

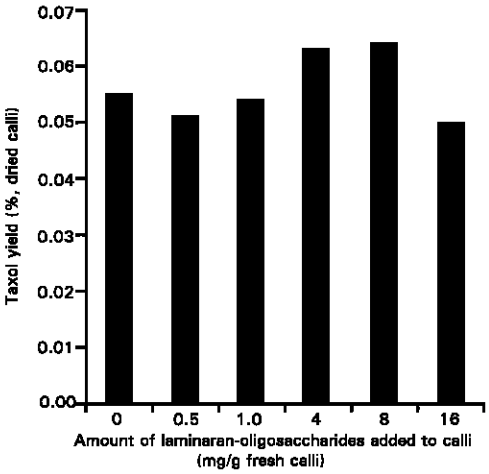

Fig. 3: Effect of laminaran-oligosaccharides on taxol production by calli from *T. cuspidata* var. nana

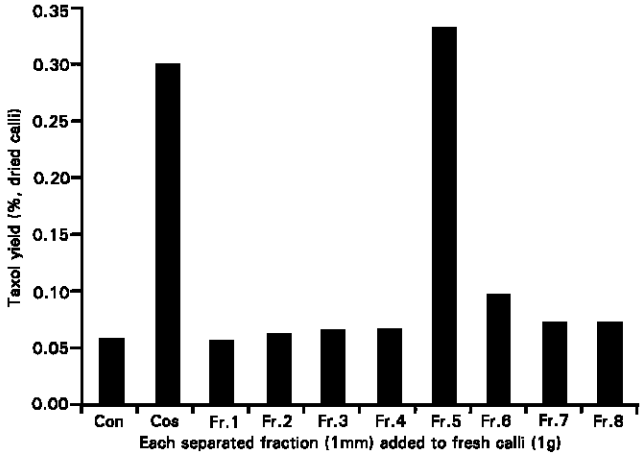

Fig. 4: Effect of each fraction of chito-oligosaccharides on taxol production by calli from *T. cuspidata* var. nana.
Con = Control
Cos = Chito-oligosaccharides obtained by a partial hydrolysis of chitosan

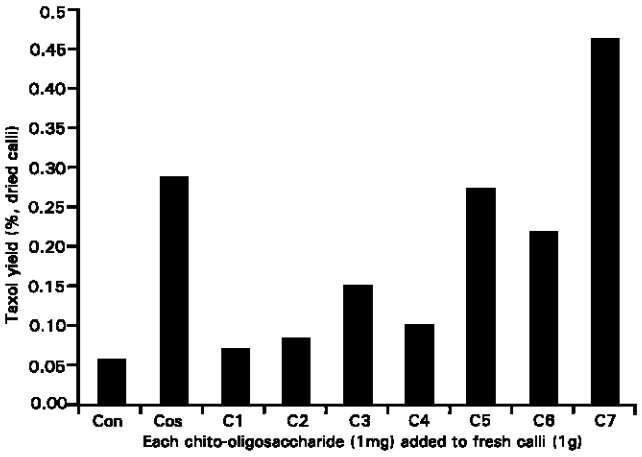

Fig. 5: Effect of each chito-oligosaccharide on taxol production by calli from *T. cuspidata* var. nana
Con = Control Cos = Chito-oligosaccharides
C1 = Glucosamine C2 = Chitobiose
C3 = Chitotroise C4 = Chitotetraose
C5 = Chitopentaose C6 = Chitohexaose
C7 = Chitoheptaose

Table 1: Effect of growth regulator on proliferation of callus derived from *Taxus cuspidate* var. nana

Combination		A	B	C
	2,4-D (mg/L)	12	8	4
	Kinetin (mg/L)	1.5	1.0	0.5
Callus growth 8 (%)		490	620	970

* Callus growth showed the growth ratios after incubation for 50 days, (%) of the starting callus as 100.

Table 2: Composition of chito-oligosaccharides and the separated fraction (Fr. 5).

Substance	Composition (%)	
	Chito-oligosaccharides	Fr. 5
Glucosamine	-	-
Chitobiose	26.9	4.3
Chitotroise	57.8	55.6
Chitotetraose	7.9	12.8
Chitopentaose	0.6	7.3
Chitohexaose	1.9	5.0
Chitoheptaose	4.9	15.0

Chito-oligosaccharides prepared by partial hydrolysis of chitosan were separated into eight fractions (Fr. 1-8) by iron-exchange chromatography.

medium), and then MS medium (45% of that on the SH medium). Fett-Neto *et al.* (1992, 1993) and Son *et al.* (2000) reported that callus was induced from *T. cuspidata* on B5 medium. Wang *et al.* (1997) and Zhang *et al.* (2001) induced callus from *T. chinensis* on MS medium. Wickremesinhe *et al.* (1993) induced callus from *Taxus* spp. on SH medium. However, comparison of growth rate of each callus induced on three media (B5, MS and SH) was not conducted. From the results obtained here, we found that there was difference in the growth rate of each callus induced on the three media.

To find the optimum concentration of 2,4-D and kinetin for the proliferation of the calli derived from the stems, three combinations [(A) – (C)] of each growth regulator were added to B5 medium. The results are shown in Table 1. Callus growth by the combination C was the most largest in the three combinations (Table 1). The callus increased 9.7-fold for 50 days. From the results obtained here, the optimum concentrations of 2,4-D and kinetin for the proliferation of the calli were 4.0 and 0.5 mg/L, respectively. In addition, B5 medium supplemented with 2,4-D and kinetin supported growth without regeneration. Subculturing on this medium led to a reduction in the proliferation of calli after five cultures and ultimately, a complete loss of proliferation after ten cultures.

In the previous paper, Tachibana *et al.* (1994) reported that callus was induced from *T. cuspidate* var. nana on MS medium supplemented with 2,4-D and kinetin. The optimum concentration of 2,4-D and kinetin for proliferation of the calli were 4.0 and 0.5 mg/L, respectively. The callus growth increased about 4 to 5-fold for 30 days. However, the calli induced on the MS medium became brown color after three subcultures and completely stopped the proliferation after five subcultures.

Production of taxol by *T. cuspidata* var. nana: The amount of taxol in each callus derived from the stems on all three media is shown in Fig. 1. The taxol in the calli derived from the stems on B5 medium constituted 0.084% dry weight, as opposed to 0.061% for the intact stems. The taxol in the calli derived from the stems on the MS and SH media constituted 0.073 and 0.029%,

respectively. The amount of taxol in the calli derived from the stems on the B5 medium was about 1.4 times higher than that in the intact plant. In addition, no taxol was found in the three agar media. In the previous paper. Tachibana *et al.* (1994) reported that the content of taxol in the calli derived from stems of *T. cuspidata* var. nana by use of MS medium was almost the same as that in the intact plant, though the amount of taxol produced by the callus cultures in MS medium was lower than that in B5 medium as mentioned above. Fett-Neto *et al.* (1992) reported that the content of taxol produced by callus cultures of *T. cuspidate* in B5 medium was almost the same as that in the intact plant. The results obtained here was almost consistent with the previous results obtained by Fett-Neto *et al.* (1992) and Tachibana *et al.* (1994). Based on growth rate and taxol yield, the B5 medium was considered to be the best for production of taxol by callus cultures of *T. cuspidata* var. nana. As the content of taxol was increased by callus cultures of *T. cuspidata* var. nana, it was considered that the production of taxol by the cultures could be enhanced by several methods, for example, treatment with an elicitor.

Effect of oligosaccharides on the production of taxol in callus cultures of *T. cuspidata* var. nana: The production of taxol was stimulated significantly when COS were added to the calli (Fig. 2). The production was stimulated 5.1-fold compared to that of the control (no COS) when 1mg of COS was added to fresh calli (1g) and the mixture incubated on B5 medium for 15 days at 25° C in the dark.

The effect of LOS on the production of taxol is given in Fig. 3. The stimulation of production was less marked on treatment with LOS than COS, with only about 1.2-fold increase over the control (no LOS).

The results show that COS stimulate the production of taxol in callus cultures of *T. cuspidata* var. nana. This is the first report on the stimulation of production of taxol in callus cultures of *Taxus* spp. by treatment with oligosaccharides. In addition, Furmanowa *et al.* (2000) reported that chitosan (a polymer of glucosamine) enhanced the taxol yield by 1.5- fold in callus cultures of *T. media.* They also reported that the production of taxol was not enhanced in callus cultures of *T. cuspidata* treated with chitosan.

Muranaka *et al.* (1998) found that the production of podophyllotoxin in callus cultures of *Juniperus chinensis* was stimulated about 15-fold when COS was added. Kobayashi *et al.* (1993) reported that LOS had elicitor activity and stimulated the production of antifungal compounds in alfalfa. However, in case of callus cultures of *T. cuspidata* var. nana, the effect of LOS on taxol production was less than that of the antifungal compounds in alfalfa. Muranaka *et al.* (1998) found that the production of podophyllotoxin by callus cultures of *J. chinensis* was stimulated about 3.5-fold when LOS was added. In the case of callus cultures of *T. cuspidata* var. nana, the stimulation of taxol production by treatment with LOS was 2.9 times lower than that of the production of podophyllotoxin. These results suggest that COS and LOS act as elicitor, though the degree to which they affect taxol production may vary.

The active compound in chito-oligosaccharides that stimulates the production of taxol: The production of taxol was stimulated significantly when Fr.5 was added (Fig. 4). The production increased about 5.7-fold over that of the control (no COS) when 1 mg of Fr.5 was added to fresh calli (1g). Fr.5 was further analyzed by HPLC. The composition of Fr.5 is shown in Table 2. The main constituents are chitotriose, chitotetraose and chitoheptaose.

Effect of each constituent in Fr.5 on stimulation of taxol production in the callus cultures was examined and the results are shown in Fig. 5. Production increased about 8.2-fold compared with control (no chito-oligosaccharide) when 1 mg of chitoheptaose was added to fresh calli (1g). The results show that chitoheptaose is the most active compound in COS for stimulation of taxol production. There is no report on the stimulation of production of taxol in callus cultures of *Taxus* spp. by treatment with chitoheptaose. We also found that chitoheptaose stimulates the production of taxol in cell suspension cultures of *T. cuspidate* var. nana. The results will be published later.

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References

Fett-Neto, A.G., F. DiCosmo, W. F. Reynolds and K. Sakata, 1992. Cell culture of *Taxus* as a source of the antineoplastic drug taxol and related taxanes. Biotechnology, 10: 1572-1575.

Fett-Neto, A.G., S. J. Melanson, K. Sakata and F. DiCosmo, 1993. Improved growth and taxol yield in developing calli of *Taxus cuspidata* by medium composition modification. Biotechnology, 11: 731-734.

Fett-Neto, A.G., S. J. Melanson, S. A. Nicholson, J. J. Pennington and F. DiCosmo, 1994a. Improved taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *Taxus cuspidata*. Biotechnol. Bioeng., 44: 967-971.

Fett-Neto, A.G., W.Y. Zhang and F. DiCosmo, 1994b. Kinetics of taxol production, growth, and nutrient uptake in cell suspension of *Taxus cuspidata*. Biotechnol. Bioeng., 44: 205-210.

Furmanowa, M., H. Oledzka, K. Sykiowska-Baranek, J. JÓzefowicz and S. Gieracka, 2000. Increased taxane accumulation in callus cultures of *Taxus cuspidata* and *Taxus* x *media* by some elicitors and precursors. Biotechnol. Lett., 22: 1449-1452.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Gelmon, K., 1994. The taxoids:paclitaxel and docetaxel. The Lancet, 344: 1267-1272.

Holton, R.A., C. Somoza, H.-B. Kim, F. Liang, R. J. Biediger, P.D. Boatman, M. Shindo, C. C. Smith, S. Kim, H. Nadizadeh, Y. Suzuki, C. Tao, P. Vu, S. Tang, P. Zhang, K. K. Murthi, L. N. Gentile and J. H. Liu, 1994a. First total synthesis of taxol. 1. Functionalization of the B ring. J. Am. Chem. Soc., 116: 1597-1598.

Holton, R.A., H.B. Kim, C. Somoza, F. Liang, R. J. Biediger, P. D. Boatman, M. Shindo, C. C. Smith, S. Kim, H. Nadizadeh, Y. Suzuki, C. Tao, P. Vu, S. Tang, P. Zhang, K. K. Murthi, L. N. Gentile and J. H. Liu, 1994b. First total synthesis of taxol. 2. Completion of the C and D rings. J. Am. Chem. Soc., 116: 1599-1600.

Holton, R.A., R.J. Biediger and P.D. Boatman, 1995. *In*: Taxol®: Science and Applications. Suffness, M.(Ed.). CRC Press, New York, pp: 97-121.

Ketchum, R.E.B., D.M. Gibson and L.G. Gallo, 1995. Media optimization for maximum biomass production in cell cultures of pacific yew. Plant Cell, Tissue and Organ Cultures, 42: 185-193.

Kikkawa, Y., T. Kawada, I. Furukawa and T. Sakuno, 1990. A convenient preparation method of chito-oligosaccharides by acid hydrolysis. J. Fac. Agric. Tottori Univ., 26: 9-17.

Kobayashi, A., A. Tai, H. Kanzaku and K. Kawazu, 1993. Elicitor-active oligosaccharides from algal laminaran stimulate the production of antifungal compounds in alfalfa. Z. Naturforsch., 48C: 575-579.

McLaughlin, J.L., R.W. Miller, R.G. Powell and C.R. JR. Smith, 1981. 19-Hydroxybaccatin III, 10-Deacetylcephalomannine, and 10-Deacetyltaxol: New antitumor taxanes from *Taxus wallichiana*. J. Nat. Prod., 44: 313-319.

Muranaka, T., M. Miyata, K. Itoh and S. Tachibana, 1998. Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. Phytochemistry, 49: 491-496.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Pl., 15: 473-479.

Nicolaou, K.C., Z. Yang, J.J. Liu, H. Ueno, P. G. Nantermet, R. K. Guy, C. F. Claiborne, J. Renaud, E. A. Couladouros, K. Paulvannan and E.J. Sorensen, 1994. Total synthesis of taxol. Nature, 367: 630-634.

Schenk, R. U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., 50: 199-204.

Son, S.H., S.M. Chou, Y. H. Lee, K. B. Choi, S. R. Yun, J. K. Kim, H. J. Park, O. W. Kwon, E. W. Noh, J. H. Seon and Y. G. Park, 2000. Large-scale growth and taxane production in cell cultures of *Taxus cuspidata* (Japanese yew) using a novel bioreactor. Plant Cell Reports, 19: 628-633.

Stierle, A., G. Strobel and D. Stierle, 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific Yew. Science, 260: 214-216.

Strobel, G., X. Yang, J. Sears, R. Kramer, R. S. Sidhu and W. M. Hess, 1996. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology, 142: 435-440.

Tachibana, S., A. Matsuo, K. Itoh and T. Oki, 1994a. Extractive in the leaves and bark of *Taxus cuspidata* Sieb. *et* Zucc. var. nana Rehder. Mokuzai Gakkaishi, 40: 1008-1013.

Tachibana, S., E. Watanabe, K. Itoh and T. Oki, 1994b. Formation of taxol in *Taxus cuspidata* Sieb. *et* Zucc. *var. nana* Rehder callus cultures. Mokuzai Gakkaishi, 40: 1254-1258.

Uehara, K., 1969. Jumoku Daizusetsu. Vol. 1, Ariake Shobo Publisher, Tokyo, pp: 36-43.

Wang, H.Q., J.J. Zhong and J.T. Yu, 1997. Enhanced production of taxol in suspension cultures of *Taxus chinensis* by controlling inoculum size. Biotechnol. Lett., 19: 353-355.

Wani, M.C., H.L. Taylor, M.E. Wall, P. Coggon and A.T. Mcphail, 1971. Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem. Soc., 93: 2325-2327.

Wickremesinhe, E.R.M. and R.N. Arteca, 1993. *Taxus* callus cultures: Initiation, growth optimization, characterization and taxol production. Plant Cell, Tissue and Organ Culture, 35: 181-193.

Witherup, K.M., S.A. Look, M.W. Stasko, T.J. Ghiorzi and G. M. Muschik, 1990. *Taxus* spp. Needles contain amounts of taxol comparable to the bark of *Taxus brevifolia*: Analysis and isolation. J. Nat. Prod., 53: 1249-1255.

Zhang, C.H. and H.B. Xu, 2001. Improved paclitaxel production by *in site* extraction and elicitation in cell suspension cultures of *Taxus chinensis*. Biotechnol. Lett., 23: 189-193.