BIOCELL
2010, 34(1): 1-6

ISSN 0327- 9545
PRINTED IN ARGENTINA

Taxus globosa S. cell lines: Initiation, selection and characterization in terms of growth, and of baccatin III and paclitaxel production

Dulce Ma. Barradas-Dermitz[1,*], Patricia M. Hayward-Jones[2], Martín Mata-Rosas[3], Beatriz Palmeros-Sánchez[2], Oscar B. J. Platas-Barradas[4], Rodolfo F. Velásquez-Toledo[2]

1. Chemical Biology Area, Instituto Tecnológico de Veracruz, México.
2. Chemical Biology Area, Universidad Veracruzana, México.
3. Plant Cell Culture Laboratory, Instituto de Ecología, A.C., Veracruz, México.
4. Biochemical Engineering Area, Instituto Tecnológico de Veracruz, México.

Key words: growth-associated metabolites, methyl jasmonate, paclitaxel precursor, secondary metabolism, suspension cell culture.

ABSTRACT: Of the initial six cell lines originating from explants of *Taxus globosa,* or Mexican yew (stem internode, leaves and meristematic tissue), three were selected for their microbial and oxidation resistance, two from leaves and the other from stem internode. A study of their behavior, both in terms of cell growth, and of baccatin III and paclitaxel production, was developed in suspension cultures with an initially standardized biomass (fresh weight 0.23 g/L) using modified Gamborg's B5 medium, and an elicitor (methyl jasmonate), on either the first or seventh day of culture, at several levels (0, 0.1, 1, 10, 100 µM). In most of the conditions used, the three cell lines showed growth associated baccatin III production. The cell line from stem internode was the highest producer of baccatin III using 1 µM elicitor, sampling at 10 days ($p \leq 0.01$, 6.45 mg/L). This same line also had the highest biomass production (6.85 g/L, $p \leq 0.01$) at 10 days of culture but at the higher elicitor concentration of 10 µM. All three cell lines did not produce paclitaxel under experimental conditions used.

Introduction

The establishment of cell lines from different species of the *Taxus* genus, their chemical biology as well as engineering studies, have been the subject of various research projects referred to in the literature (Zhong, 2002; Suffness, 1995). These scientific and engineering activities have been mainly driven by the search for alternative sources of paclitaxel, a diterpenoid originally isolated by Wani *et al.* (1971) from the bark of the Pacific yew, *Taxus brevifolia* Nutt (Taxaceae). Paclitaxel has been recognized by the National Cancer Institute-USA, as the "most well-known natural-source cancer drug in the USA".

Taxus globosa S.*,* the Mexican yew, is one of the eight species included in the genus (Appendino, 1995). A comparative analysis of taxane diterpenoid contents made on samples of different *Taxus* species showed that *T. globosa* and *T. floridana* have the highest paclitaxel content (400-500 µg/g dried needles) and *T. celebica* or *T. chinensis* the lowest (26 µg/g dried needles) (van Rozendaal *et al.,* 2000). Regarding publications that include research about *Taxus globosa* cell lines (plant cell culture), as far as we know, there is only one, a patent

*Address correspondence to: Dulce Ma. Barradas-Dermitz.
E-mail: *dmbarradasd@yahoo.com*
Received: April 21, 2009. Revised version received: November 21, 2009. Acepted: December 10, 2009.

(Bringi *et al.,* 1995) where these authors also working with different *Taxus* species, found *T. globosa* to be the lowest paclitaxel producer (0.0003% dry weight) and *T. chinensis* the highest (0.18% dry weight). It is highly probable that the Bringi *et al.* (1995) findings might have been one of the factors that originated the lack of research into establishing *T. globosa* cell lines, along with their corresponding chemical biology studies. It is therefore the purpose of this work to present the results achieved by our group relating to *T. globosa* cell lines.

Current industrial processes for paclitaxel production involve mainly two routes. One of them extracts 10-deacetyl baccatin III (10-DAB) the paclitaxel precursor which is abundant in some *Taxus* species needles (e.g., *T. baccata* (English yew), *T. wallichiana*) and either transforms it into paclitaxel by Holton's 4 step synthesis (metal alcoxide process, Holton, 1992; Holton *et al.*, 1997), or uses it for the semisynthesis of a paclitaxel analog: docetaxel or taxotere (a non-natural derivative). The other route is by plant cell culture (*T. canadensis, T. cuspidata, T. chinensis*).

Efforts to find more efficient processes (higher paclitaxel yields with improved sustainability), as well as basic research on *Taxus* species*,* are going on in different countries. As far as we can ascertain, in the case of Latin America, Goleniowski (2000) and our group are pioneering this line of research.

Materials and Methods

Explants. Establishment and culture of callus lines

Branch fragments, five cm in length and approximately 2 mm in diameter, of the three *Taxus globosa* trees located in the Francisco Javier Clavijero Botanical Gardens (Instituto de Ecología, A.C., Xalapa, Ver. Mexico) were collected and immediately processed. The first part of the procedure involved: washing with liquid soap aqueous solution (30 min with agitation); rinsing in tap water; washing with fungicide solution (Benlate^{TM}, 3 g/L), 2 h with agitation; decantation; surface-sterilised with 70% ethanol for 1 min, followed by 30% (v/v) sodium hypochlorite, 35 min. The explants were rinsed four times with sterile distilled water under aseptic conditions.

The explants from leaves, stem internode and meristematic tissue (lateral vegetative bud), were placed on medium containing Murashige and Skoog salts and vitamins (Murashige and Skoog, 1962), glycine 2 mg/L, myo-inositol 100 mg/L, sucrose 30 mg/L and Woody Plant medium (Lloyd and McCown, 1980). The pH in all media were adjusted to 5.6 with NaOH and HCl 0.1 N before adding 8g L^{-1} agar (Sigma Chemical Co., St. Louis, MO) and autoclaving at 120ºC for 15 min. The plates were incubated at 25 ± 1ºC, half of them in the dark, the rest under a 16 h photoperiod and a photonic flux of 20 µmol $m^{-2} s^{-1}$ (shade conditions). Light conditions for the photoperiod were supplied by fluorescent lighting at 1500 watts. There were three subcultures in total to new solid medium every 3 months. Aseptic callus cell lines without extensive browning were selected for further experiments.

Cell suspension cultures from callus, and its selection

For the first suspension cultures, callus chunks from those selected were aseptically transferred to 100 ml Murashige and Skoog liquid medium in 150 ml Erlenmeyer flasks. They were incubated for 92 days (140 rpm, 25ºC), half of them in the dark, the rest under a 16 h photoperiod and a photonic flux of 20 µmol $m^{-2} s^{-1}$. For all of the following transfers of suspension culture to new fresh medium, each sample of 50 ml of the original culture was inoculated into a 250 ml Erlenmeyer flask containing 100 ml MS liquid medium. Average incubation time was 42 days for each of the six transfers that were made (agitation rate 140 rpm, 25ºC). After the sixth transfer, all the cell lines were stored for one month at 4ºC. Those cell lines that resisted microbial contamination were selected for the following studies.

Elicitation studies

The basic medium used for these studies was 12 ml modified Gamborg's B5 liquid medium (Gamborg *et al.,* 1968) supplemented with 2 x B_5 vitamins, 2% w/v sucrose, 0.55 mg/L (2.5 x 10^{-3} mM) 2,4-D, 100 mg/L myo-inositol and 30 mg/L glycine, pH 5.6, in a 125 ml Erlenmeyer flask, covered with a silicone foam cap (Bellco). The elicitor was methyl jasmonate (Sigma Chemical Co., St. Louis, Missouri), at four levels (0.1, 1, 10 and 100 µM) plus the control; the addition of the elicitor was made aseptically in a laminar flow cabinet, by means of a 0.22 µm GHP syringe filter (Pall), on the first or seventh culture day (since no statistical differences were observed between both timings, the results were pooled for presentation). The cultures were placed in a rotary shaker (New Brunswick) at 110 rpm, at room temperature and in darkness. Total culture was for thirty days, sampling every 10 days. An initial standardized biomass (0.23 g/L) was used.

Determination of growth (fresh biomass weight or fresh weight)

Growth was measured by changes in fresh weight. In a laminar flow cabinet, 1.0 ml suspension culture from a thoroughly stirred medium was sampled and put in an already weighed Eppendorf tube. The tube was centrifuged (14,000 rpm, 10 min), its content decanted and the pellet weighed.

Extraction procedures and baccatin III and paclitaxel determinations by HPLC

In aseptic conditions, at the end of 10, 20 and 30 days, 1.0 ml of suspension culture was sampled from a thoroughly stirred medium. Then, in a fume hood environment, this sample was ground and extracted using an electronic homogenizer (Glas Col, with teflon pestle and glass tube, 3200 rpm). A triple extraction was performed using 1.0 ml CH_2Cl_2 (J. T. Baker) each time, for 60, 30 and 15 sec respectively.

The combined organic phases were dried using ultrapure nitrogen stream, 1.0 ml MeOH HPLC grade (Burdick and Jackson) was added and microfiltered through a 0.45 µm GHP syringe filter (Pall) into an Eppendorf tube. This methanolic solution was then analyzed by HPLC (GBC), using a 3 µm Alltima Phenyl 53 x 7 mm column (Alltech), UV detector at 230 nm, mobile phase: 0.05 M TFA: MeOH: CH_3CN (50:19:31 v/v/v), flow rate 1.5 ml/min, against external standard curves for baccatin III (Sigma) and paclitaxel (Sigma).

Statistics

Using SPSS 8.0 software, data were analysed with a one-way ANOVA and *post hoc* Duncan comparison tests ($p \leq 0.05$, $p \leq 0.01$) on those cases showing significant differences, in order to pinpoint the conditions

TABLE 1.

Fresh weight (FW, g/L) and baccatin III (B, mg/L) production of three *Taxus globosa* cell lines (mean ± SD, N=4), using methyl jasmonate as elicitor, and after 0, 10, 20 and 30 days of culture; statistically significant differences between elicitor levels and culture day ($p \leq 0.01$) are denoted in bold face.

Elicitor level	Day	CL1		CL2		CL3	
		FW±SD	B±SD	FW±SD	B±SD	FW±SD	B±SD
0	0	0.23±0	0±0	0.23±0	0±0	0.23±0	0±0
0	10	1.91±0.53	1.51±1.27	2.59±1.03	1.67±1.54	3.04±1.81	1.14±1.28
0	20	2.34±0.89	1.54±1.39	2.44±1.09	1.34±1.2	3.18±1.52	1.51±1.53
0	30	2.58±0.63	1.81±2.07	2.24±1.1	1.33±1.57	3.74±1.67	1.26±1.84
0.1	0	0.23±0	0±0	0.23±0	0±0	0.23±0	0±0
0.1	10	2.35±0.48	1.15±0.52	2.93±1.01	1.65±1.1	2.95±0.26	1.46±0.21
0.1	20	3.85±0.66	2.08±0.27	4.38±0.64	1.84±0.75	3.73±0.22	1.43±1.31
0.1	30	3.5±0.7	4.87±0.66	3.98±0.73	2.51±0.58	3.55±0.88	0.98±0.71
1	0	0.23±0	0±0	0.23±0	0±0	0.23±0	0±0
1	10	2.95±0.83	**4.92±2.66**	2.33±0.37	**4.53±0.63**	2.8±0.26	**6.45±2.06**
1	20	1.35±0.21	**4.28±0.66**	1.55±0.91	**2.88±1.36**	3.03±2.72	**3.71±1.2**
1	30	2.38±0.1	**4.08±0.33**	2.2±0.4	3.11±0.99	2.43±0.21	**3.94±0.47**
10	0	0.23±0	0±0	0.23±0	0±0	0.23±0	0±0
10	10	1.85±0.58	0.97±0.72	1.95±0.21	1.08±0.46	**6.8±1.54**	0.55±0.64
10	20	3.38±3.09	0.91±0.29	1.93±0.79	0.14±0.28	4.98±1.41	0.48±0.42
10	30	1.58±0.51	0.08±0.16	3.78±3.48	0.25±0.49	4.88±1.67	0.42±0.49
100	0	0.23±0	0±0	0.23±0	0±0	0.23±0	0±0
100	10	1.93±0.82	1.46±0.99	1.73±0.55	0.46±0.53	1.93±0.82	0.73±0.56
100	20	2.48±1.15	0.27±0.55	2.2±0.08	0±0	2.48±1.15	0.83±0.18
100	30	2.4±0.37	0±0	2.18±0.59	0±0	2.4±0.37	0.06±0.12

involved. A Spearman rank test ($p \leq 0.05$) was applied to all results (fresh weight, paclitaxel and baccatin III production) to investigate any possible associations.

Results

Effect of conditions on callus initiation from explants: cell line generation

Explants of leaf, stem internode and meristematic tissue from *Taxus globosa* trees were originally inoculated on solid medium (Murashige and Skoog´s or Woody Plant medium). While most of the samples induced callus, microbial contamination or oxidation of the unorganized cells (colored callus) limited the number of cell lines selected at this level; finally six of them, all originated in Murashige and Skoog medium, were chosen. Of these six cell lines, two of them were from leaf, two from stem internode and two from meristematic tissue. Half of them were cultured in the dark, and the rest under a 16 h photoperiod.

Selection of cell lines

Callus tissues from the six cell lines were transferred to Murashige and Skoog liquid medium in order to obtain suspension cultures. After six subcultures of each line, the final suspension cultures were stored at 4ºC. Three lines were chosen using as selection criteria their resistance to microbial contamination under this storage condition.

The selected lines were named CL1, CL2 and CL3. The first two originated from leaf explants and the third from a stem internode. Meristematic lines did not show a similar resistance to microbial contamination. The difference between CL1 and CL2, both originating from leaves, is that CL1 comes from callus grown under shade conditions and CL2 under darkness conditions. CL3, the cell line from stem internode, comes from a callus grown under darkness conditions.

Effect of elicitor concentration on growth, baccatin III and paclitaxel productions

The studies were carried out in suspension cultures using modified Gamborg's B5 medium. Table 1 shows the changes in growth (fresh biomass weight) and baccatin III production for each of the three cell lines, as well as the effect of four levels of methyl jasmonate. Information from Table 1, plus a comparison of Figs. 1 and 2, show that there is an overall correlation between growth and baccatin III production under the effect of 1 μM methyl jasmonate, particularly after 10 and 20 days of culture.

Table 1 also shows the results obtained with other levels of the elicitor, and one may conclude that higher levels may be even inhibitory of baccatin III production, and that a correlation between biomass and baccatin production may not be observed under their influence.

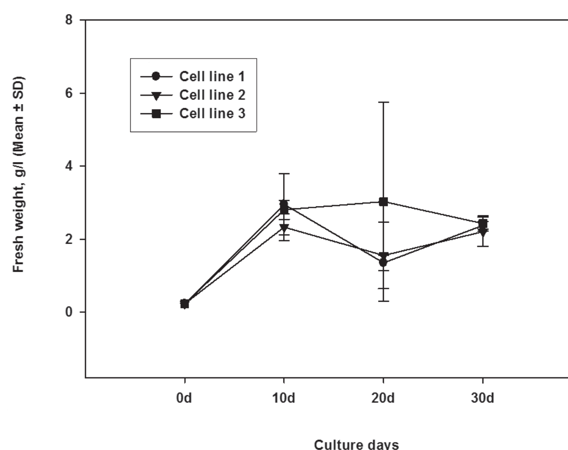

FIGURE 1. Growth of three *Taxus globosa* cell lines exposed to 1 μM of methyl jasmonate.

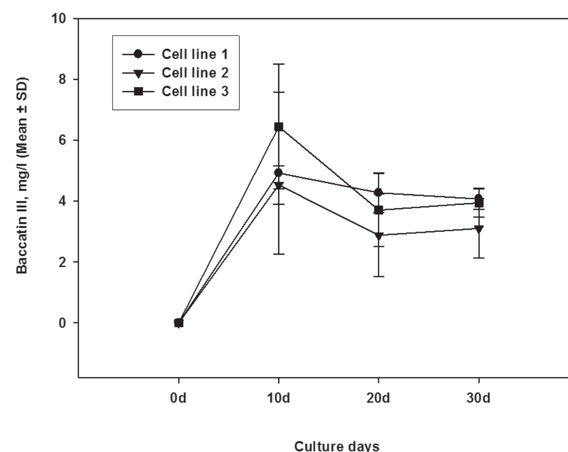

FIGURE 2. Baccatin III production by three *Taxus globosa* cell lines exposed to 1 μM of methyl jasmonate.

Discussion

The correlation found between cell growth and baccatin III production, particularly after 10 and 20 days of culture, is not a usual finding in other plant cell cultures where secondary metabolite production does not usually follows changes in biomass (Fett-Neto *et al*., 1994; Suffness, 1995; Cusidó *et al*., 2002).

Cusidó *et al.* (2002) working with *Taxus media* in 175 ml shake flask with 10 ml production media and methyl jasmonate (220 μg/g fresh weight), using an inoculum of 100 g/L cell fresh weight (436 times our inoculum size), reported at 10 days of culture, approximately 3 mg/L baccatin III; 7 mg/L was the highest production achieved on the sixteenth day. Yukimune *et al.* (1996) reported baccatin III production of 25.2 ± 2.6 mg/L by *T. media* and 53.6 ± 2.2 mg/L by *T. baccata*, after 14 days, using 100 μM methyl jasmonate, 100 ml shake flask with 20 ml production medium and using an inoculum of 125 g/L (545 times our inoculum size).

The origin of CL3 (a stem internode explant), could probably be a causative factor in its behavior in terms of higher cell growth and baccatin III production, compared to CL1 and CL2 (both of them originating from leaf explants), considering that there is experimental evidence that the nature of an explant can exert specific behavior on secondary metabolism (Banthorpe, 1994; Wewetzer, 1998; Curino *et al.*, 2001).

As we have already noted, the patent of Bringi *et al.* (1995) is the only published document on research done on *T. globosa* cell culture. However, we are unable to compare our results obtained for baccatin III production by CL1, CL2 and CL3 because only paclitaxel results for this *Taxus* species were included in this patent. Our paclitaxel results were insufficient to be able to make meaningful statistical analyses, seeing as under the conditions studied, cell lines 1, 2 and 3 could be classified as paclitaxel nonproducers, a behavior already seen in other *Taxus* species (Kim *et al.*, 2004).

An observation which is noteworthy is the apparent contradiction existing between the approximately 19-fold higher paclitaxel content found in needles of *T. globosa,* or Mexican yew (400-500 μg/g dried needles; van Rozendaal *et al.,* 2000) compared with *T. celebica* or *T. chinensis* (26 μg/g dried needles), and the paclitaxel production obtained in plant cell culture from these species: Bringi *et al.* (1995) found *T. globosa* to be the lowest paclitaxel producer (0.0003% dry weight) and *T. chinensis* the highest (0.18% dry weight). In our study, as mentioned above, *T. globosa* CL1, CL2 and CL3 can be considered as paclitaxel nonproducers, under the conditions used.

From the results of research work carried out to elucidate paclitaxel biosynthetic pathways, it can be seen that both a carbon and a nitrogen precursor are needed. Walker and Croteau (2001), Croteau *et al.* (1995), Floss and Mocek (1995), and Fleming *et al.* (1994), have suggested that in this process there are terminal steps that originate the paclitaxel C-13 side chain, in which phenylisoserine and baccatin III are the nitrogen and carbon precursors respectively, whose interaction generate the esterification of baccatin C-13 hydroxyl reaction, followed by that responsible for the N-benzoylation of the side chain. This scheme clearly implicates baccatin III as a paclitaxel precursor molecule. Therefore, the irregular paclitaxel production observed in the three *T. globosa* cell lines and their evident baccatin III production, open up several possibilities, one of them a deficiency of the nitrogen precursor.

In contrast, Srinavasan *et al.* (1996) established both a cytoplasmic and a plastidic origin for baccatin III production by *T. chinensis* PRO1-95, and in the case of paclitaxel only a plastidic one. This finding, with the support of other results, led them to suggest that baccatin III "may not be a direct precursor" of paclitaxel. The argument of Srinavasan *et al.* could also explain the observed behavior of *T. globosa* cell lines CL1, CL2, CL3, in terms of differentiated baccatin III and paclitaxel production responses.

Both these views of the baccatin III / paclitaxel relationship need further research on the three *T. globosa* cell lines studied, as well as studies of the significance of different explant sources, physiological age and culture media amongst others.

References

Appendino G (1995). The phytochemistry of the yew tree. *Natural Product Reports* **12**: 349-360.

Banthorpe DV (1994). Secondary metabolism in plant tissue culture; scope and limitations, *Natural Products Reports*, 303-328.

Bringi V, Kadkade PG, Prince CL, Schubmehl BF, Kane EJ, Roach B (1995). Enhanced production of taxol and taxanes by cell cultures of *Taxus* species. U.S. Patent No. 5 407 816, Asignee: Phyton Catalytic, Inc.

Croteau R, Hezari M, Hefner J, Koepp A, Lewis NG (1995). Paclitaxel biosynthesis. In *Taxane anticancer agents, basic science and current status*. Gerog G I, Chen TT, Ojma I, Vyas DM Eds; ACS Symposium Series 583; *American Chemical Society*, Washington, D.C. pp. 72-80.

Curino A, Milanesi L, Benassati S, Skliar M, Boland R (2001). Effect of culture condition on the synthesis of vitamin D3

metabolites in *Solanum glaucophyllum* grown *in vitro*. *Phytochemistry* **58**: 47-52.

Cusidó RM, Palazón J, Bonfill M, Navia-Osorio A, Morales C, Piñol MT (2002). Improved paclitaxel and baccatin III production in suspension cultures of *Taxus media*. *Biotechnology Progress* **18**: 418-423.

Fett-Neto AG, Zhang WY, DiCosmo F (1994). Kinetics of Taxol production, growth and nutrient uptake in cell suspensions of *Taxus cuspidata*. *Biotechnology and Bioengineeering* **44**: 205-210.

Fleming PE, Knaggs AR, He XG, Mocek U, Floss HG (1994). Biosynthesis of taxoids. Mode of attachment of taxol side chain. *Journal of the American Chemical Society* **116**: 4137-4138.

Floss HG, Mocek U (1995). Biosynthesis of taxol. In *Taxol: science and applications*. Suffness M Ed.; CRC Press: Boca Raton, FL, pp. 191-208.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**: 151-158.

Goleniowski ME (2000). Cell lines of *Taxus* species as source of the anticancer drug Taxol. *Biocell* **24**: 139-144.

Holton RA, Somoza C, Kim HB, Shindo M, Biediger RJ, Boatman PD, Smith CH, Liang F, Murthi K (1997). Tricyclic and tetracyclic taxanes. U.S. patent number 5 637 732. Assignee: Florida State University.

Holton RA (1992). Method for preparation of Taxol using beta lactam.U.S. patent number 5 175315. Assignee: Florida State University.

Kim B, Gibson DM, Shuler M (2004). Effect of subculture and elicitation on instability of taxol production in *Taxus* sp. suspension cultures. *Biotechnology Progress* **20**: 1666-1673.

Lloyd G, Mc Cown B (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Proceedings of the International Plant Propagation Society* **30**: 421-427.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plantarum* **15**: 473-497.

Srinavasan V, Ciddi V, Bringi V, Shuler ML (1996). Metabolic inhibitors, elicitors and precursors, as tools for probing yield limitation in taxane production by *Taxus chinensis* cell cultures. *Biotechnology Progress* **12**: 457-465.

Suffness M (ed.) (1995). *Taxol: science and applications*. CRC Press, Boca Raton, Florida.

van Rozendaal ELM, Lelyveld P, van Beek TA (2000). Screening of the needles of different yew species and cultivars for paclitaxel and related taxoids. *Phytochemistry* **53**: 383-389.

Walker K, Croteau R (2001). Taxol biosynthetic genes, *Phytochemistry* **58**:1-7.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971). Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia* *Journal of the American Chemical Society* **93**: 2325-2327.

Wewetzer A (1998). Callus cultures of *Azadyrachta indica* and their potential for the production of azadirachtin, *Phytoparasitica* **26**: 47-52.

Yukimune Y, Tabata H, Higashi Y, Hara Y (1996). Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nature Biotechnology* **14:** 1129–1132.

Zhong JJ (2002). Plant cell culture for production of paclitaxel and other taxanes. *Journal of Bioscience and Bioengineering* **94** : 591-599.