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Taxus callus cultures: Initiation, growth optimization, characterization and taxol production

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Abstract

Callus was induced from *Taxus baccata* cv. Repandens Parsons ex Rehd., *T. brevifolia* Nutt., *T. cuspidata* Sieb. & Zucc., and *T.* × *media* cvs. Hicksii and Densiformis Rehd. using different concentrations of 2,4-D-(2,4-dichlorophenoxyacetic acid), IBA (indole-3-butyric acid), or NAA α-naphthalene acetic acid in combination with kinetin. All cultures grew slowly following the first subculture, and a majority turned brown and ceased growth within the next six to twelve months. The callus cultures which lived, continued to grow very slowly for one to two years before the growth rate improved. Initiation of roots and shoot primordia-like structures occurred on some cultures maintained in the dark, and 16 h light/8 h dark, respectively. A fast-growing, habituated callus line (CR-1) derived from *T.* × *media* Rehd. cv. Hicksii was established from callus initiated in 1986. Supplementing the medium with casein hydrolysate and both fructose and glucose enhanced the growth rate. A great deal of heterogeneity was found among and within the callus, with respect to the amount of taxol produced. The callus exhibited levels of taxol ranging from 0.1 to 13.1 mg kg^{-1} (0.0001 to 0.0131%) on a dry weight basis. Overall, the older brown-colored callus produced more taxol than the younger pale yellow-colored callus. The presence of taxol in callus samples was established by high performance liquid chromatography, its biological activity confirmed by a microtubule-stabilizing bioassay and its structure confirmed using one- and two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, IBA – indole-3-butyric acid, NAA – α-naphthaleneacetic acid, kinetin – 6-furfurylaminopurine, 2iP – 6-(γ,γ-dimethylallylamino)purine

Introduction

Taxol (NSC-125973) is a cytotoxic diterpene initially isolated from the stem bark of *Taxus brevifolia* Nutt. (Wani et al. 1971) and is a potent inhibitor of cell replication, blocking cells in the G2/M phase of the cell cycle (Horowitz et al. 1986). It is the only plant secondary metabolite known to promote the assembly of microtubules and inhibit the tubulin disassembly process (Schiff et al. 1978; Horowitz et al. 1986), and thus appears to be the prototype of a new class of cancer chemotherapeutic agents (Suffness & Cordell 1985).

Taxol has shown excellent activity in both phase I and phase II trials in the treatment of advanced, progressive, and drug-refractory ovarian cancer (McGuire et al. 1989; Einzig et al. 1991; Markman 1991). Most recently, it was reported that taxol had striking results in the

treatment of metastatic breast cancer (Holmes et al. 1991) and significant activity in the treatment of non-small cell lung cancer (Ettinger 1992). The limited supply of taxol has precluded extensive clinical studies with other cancers.

To date, the sole source of taxol has been the bark of *T. brevifolia*. Based on the current bark-extraction procedures, 7200 kg of bark are needed to produce one kg of taxol, and the projected target for 1993 is 230 kg (Stull 1992). As of July 1992, taxol has been made available to more than 1700 patients with refractory ovarian cancer (Arbuck 1992).

Chemists all over the world have been involved in an attempt to synthesize taxol; however, total synthesis has not been achieved (Kingston 1991; Wender 1992). However, a protocol for the semi-synthesis of taxol from baccatin III has been reported (Holton 1992).

Taxol has been identified in several other species of yew (genus *Taxus*, family Taxaceae). These include *T. baccata*, *T. cuspidata*, *T. canadensis* and *T.* × *media* cvs. Densiformis and Hicksii (Wani et al. 1971; Videnšek et al. 1990; Witherup et al. 1990; Wickremesinhe 1992).

Plant tissue culture is one of the approaches available to provide large amounts and a stable supply of compounds exhibiting antineoplastic activity, and has been of major interest since the 1970's. The production of antineoplastic compounds in callus cultures from a number of antineoplastic agent-producing plants have been demonstrated (Misawa et al. 1985; Stafford 1991).

Many scientists and pharmaceutical companies are currently pursuing tissue culture as a viable alternative for producing taxol and related taxanes. A process to produce taxol or taxol-like compounds in cell culture has already been patented (Christen et al. 1991), and most recently, taxol production was reported in 2 to 6 month old callus cultures of *T. cuspidata* (Fett-Neto et al. 1992).

The development of fast-growing cell lines capable of producing taxol (via plant tissue culture techniques) would not only solve the limitation in taxol supplies presently needed for clinical use, but will also help conserve the large number of trees that need to be harvested in order to isolate it.

The objective of this study was to induce and establish callus cultures from different *Taxus* plants, to evaluate the nutrient requirements and culture conditions in order to maximize and sustain continuous growth over long periods of time, to evaluate callus for the production of taxol, and to demonstrate the presence of taxol by HPLC, microtubule-stabilizing bioassay and NMR spectroscopy.

Materials and methods

Plant material

T. baccata cv. Repandens Parsons ex Rehd., *T. cuspidata* Sieb. & Zucc., and *T.* × *media* cv. Hicksii Rehd. growing on The Pennsylvania State University, University Park, campus, and *T. brevifolia* Nutt., and *T.* × *media* cv. Densiformis Rehd. plants obtained from a commercial nursery and established in the greenhouse were used as explant sources.

Induction of callus cultures

Stems and needles from current seasons growth were used as explants. Stems were stripped of needles and the stem sections (2.5 to 4.0 mm in diameter) and needles were surface sterilized by immersing in a solution of 1.1% sodium hypochlorite (20% commercial bleach) for 20 min, rinsed three times with sterile distilled water, and aseptically dissected into explants 5 to 7 mm in length.

B5 medium (Gamborg et al. 1968) supplemented with 2 × B5 vitamins, and 20 g l^{-1} sucrose was used with either 2,4-D, IBA, or NAA at concentrations ranging from 0.1 to 10.0 mg l^{-1} in combination with either 0.2 or 2.0 mg l^{-1} kinetin (all media components purchased from Sigma Chemical Co., St. Louis, Missouri, USA).

The pH of the medium was adjusted to 5.6 with 1 N potassium hydroxide, and Gelrite (Scott Laboratories, Inc., West Warwick, Rhode Island, USA) added at 2 g l^{-1}. The medium was autoclaved at 121°C for 15 min, and aseptically poured into sterile petri plates. Explants were placed on the solidified nutrient medium (5

explants per petri plate), and incubated in total darkness or under a 16 h day (cool white fluorescent light, 35 $\mu mol\, m^{-2}\, s^{-1}$) and 8 h dark day/night regime, and 25°C.

A habituated callus culture of *T.* × *media* cv. Hicksii was initiated by subculturing callus cultures onto medium without plant growth regulators in 1986 (Wickremesinhe 1992). This habituated callus line (CR-1) exhibited significantly faster growth compared to other callus lines we have established. This paper will focus mainly on studies performed with this particular callus line, mainly because it was fast-growing and therefore produced masses of callus needed for all the described experiments.

Media compositions and supplements

The basal medium was supplemented with either casein hydrolysate (0.1 and 1.0 $g\, l^{-1}$) or a combination of both arginine and glutamine (0.1 and 1.0 mM each) in an effort to increase callus growth. Media pH was adjusted to a range between 4.0 to 7.0 (before autoclaving) to study the effect of pH on callus growth. All experiments were performed in petri plates containing solid medium and each plate was inoculated with 500 to 750 mg of callus. Each treatment was replicated at least ten times.

The effect of fructose and glucose (10 to 40 $g\, l^{-1}$) was evaluated both with and without the presence of sucrose on the fast-growing CR-1 callus already cultured in the presence of 1.0 $g\, l^{-1}$ casein hydrolysate. Finally, CR-1 callus growth on B5 medium was compared with MS, and WP (Lloyd & McCown 1980) medium in the presence of 2 × B5 vitamins, 20 $g\, l^{-1}$ sucrose, 2.5 $g\, l^{-1}$ each of glucose and fructose, and 1.0 $g\, l^{-1}$ casein hydrolysate.

Growth measurements

Initially, callus were cultured in petri plates and subcultured every six to eight weeks. Selected 'faster growing' callus were grown in Magenta GA7 vessels containing 100 ml of solid improved nutrient medium as defined above. Growth measurements were made by aseptically weighing the initial callus inoculum and the callus mass after five weeks in culture. Membrane rafts (Sigma Chemical Co., St. Louis, Missouri, USA) placed inside Magenta GA7-3 vessels containing 45 ml of liquid medium were also used routinely for growing CR-1 callus. Growth rate studies comparing the three basal media formulations were performed using three grams of callus as the initial innoculum per raft. The use of membrane rafts facilitated the monitoring of callus growth by being able to aseptically weigh the rafts individually without disturbing the callus. Each of these treatments were replicated at least 8 times and repeated twice.

Mitotic index

CR-1 callus samples belonging to different ages in culture were stained with a mixture consisting of 0.5% glutaraldehyde and 1 $\mu g\, ml^{-1}$ Hoechst 33258 in PBS (phosphate buffered saline, pH 7.2) and viewed under UV excitation. The number of nuclei in mitosis – late prophase to late anaphase, depicting condensed chromosomes–were counted from a field of at least 500 cells. The mitotic index was calculated as the percentage of the number of nuclei in mitosis to the total number of nuclei scored.

Extraction from callus

Callus samples harvested for analysis were freeze dried and extracted (50 mg dry weight per sample) in methanol (1.5 ml) by homogenizing in an Omni-mix homogenizer (Omni International, Waterbury, Connecticut, USA) for 2 min followed by sonication for 5 minutes. The extract was transferred to a 2-ml Eppendorf tube and centrifuged to pellet cell debris. The supernatant was recovered, filtered through a 0.2 micron nylon filter, and the final volume was adjusted to 1.5 ml with methanol.

High Performance Liquid Chromatography (HPLC)

Analytical HPLC analysis was performed on a Dynamax 60 Å 8 μm phenyl column (4.6 mm × 250 mm) with a phenyl guard module (Rainin Instrument Co. Inc., Woburn, Massachusetts, USA). A mobile phase consisting of methanol:50 mM acetate buffer (pH =

4.4):acetonitrile (20:41:39), at a flow rate of 1 ml per minute was used. Taxol was detected by monitoring absorbance at 227 nm.

Duplicate injections (25 and 50 µl each) were made from every sample (extract) and the average of the two peak areas was used to quantify taxol concentration. The detection limit of taxol was 5 nanograms per injection. Authentic taxol was obtained from Dr. Mathew Suffness (National Cancer Institute, Bethesda, Maryland, USA).

Taxol was purified from CR-1 callus cultures using a 65% methanol:chloroform partitioning followed by C_{18} bonded silica gel column chromatography, and finally by C_{18} preparatory HPLC as described by Wickremesinhe & Arteca (1993a).

Microtubule-stabilizing bioassay

Neuronal microtubule proteins were prepared according to the methods of Shelanski et al. (1973) using fresh calf brains. Microtubule-associated proteins were removed (Hamel & Lin 1981), and the last tubulin pellet was taken up in cold PM buffer (50 mM 1,4-piperazinediethanesulfonic acid [Pipes; Sigma Chemical Co., St. Louis, Missouri, USA], pH 6.9; 1 mM magnesium sulfate; 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid [EGTA]) according to Cyr & Palevitz (1989). This was further purified by phosphocellulose chromatography according to Weingarten et al. (1975) to remove endogenous microtubule associated proteins (MAP's).

Taxol purified from CR-1 callus was resuspended in PM buffer. For the dark-field assay, equimolar ratios of taxol and tubulin (10 µM) were used. Authentic taxol, and PM buffer were used as controls. All test mixtures were incubated at 37°C for 15 min and examined under a Reichert Zetopan microscope (American Optical Co., Buffalo, New York, USA) equipped with a dark-field condenser and a 100 watt mercury lamp. The microtubules were placed at 4°C for 15 min and immediately fixed with 1% glutaraldehyde in order to exhibit cold-stablity.

Nuclear Magnetic Resonance spectroscopy (NMR)

Authentic taxol and taxol purified from CR-1 callus cultures were solubilized in deuterated methylene chloride and the ^{1}H and ^{13}C NMR spectra were analyzed by one- and two-dimensional methods as described by Falzone et al. (1992).

Results and discussion

Initiation of callus cultures

Callus was induced from explants derived from all five sources, i.e., *T. baccata* cv. Repandens, *T. brevifolia*, *T. cuspidata*, and *T.* × *media* cvs. Hicksii and Densiformis. Young stems were the best source of explants, compared to needles and mature stems. Explants obtained from greenhouse grown plants were less contaminated with bacteria and/or fungi (4%), compared to the explants obtained from mature outdoor trees and shrubs (up to 20%).

A wide range of auxin (2,4-D, NAA, and IBA) concentrations in combination with 0.2 mg l^{-1} kinetin induced callus (Table 1). The treatments containing 2.0 mg l^{-1} kinetin either did not produce any callus and killed the explant or produced significantly smaller amounts of callus compared to the corresponding treatments containing 0.2 mg l^{-1} kinetin. The effect of either auxin or kinetin alone was not evaluated. Callus initiation occurred on more than 75% of all the immature stem explants within 2 to 3 weeks (Fig. 1a) after being placed in the presence of the best auxin and kinetin concentrations (Table 1).

Initial callus induction occurred from the cut surface of the stems and any areas from where the needles were stripped off. However, a majority of the callus appeared to originate from an area within the stem which resulted in splitting and peeling of the epidermis and related stem tissues due to the growing callus. Although some of the other hormonal regimes also induced callus, the callus itself was comparatively slow-growing and much smaller in mass.Overall, 2,4-D

Table 1. Rating of callus induction from *Taxus* explants (derived from immature stems), placed Gamborg's B5 salts supplemented with 2 × B5 vitamins, 20.0 g l^{-1} sucrose and either 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), or α-naphthaleneacetic acid (NAA) ranging from 0.2 to 10.0 mg l^{-1} in combination with 0.2 mg l^{-1} 6-furfurylaminopurine (kinetin) and solidified with 2.0 g l^{-1} gelrite. Each observation is based on 50 to 200 explants incubated in total darkness for 4 weeks. (0 = no callus, 1 = less than 30% of the explants produced callus, 2 = 30 to 75% of the explants produced callus, 3 = greater than 75% of the explants produced callus).

Taxus source	2,4-D(mg l^{-1})					NAA (mg l^{-1})				IBA (mg l^{-1})		
	0.2	0.5	1	2	5	0.5	2	5	10	2	5	10
Taxus brevifolia	1	2	3	3	1	0	2	1	1	1	1	0
T. baccata cv. Repandens	0	1	3	2	1	0	0	1	1	0	2	1
T. cuspidata	0	0	1	1	0	0	2	3	2	0	1	1
T. × *media* cv. Densiformis	0	1	2	1	0	1	3	3	1	1	1	0
T. × *media* cv. Hicksii	0	1	3	1	0	1	3	2	2	1	1	0

was the better source of auxin compared to NAA and IBA for both *T. brevifolia* and *T. baccata* cv. Repandens, while NAA was better for both *T. cuspidata*, and *T.* × *media* cv. Densiformis. However, with *T.* × *media* cv. Hicksii, NAA and 2,4-D exhibited similar rates of success, and was better than IBA (Table 1) .

Callus cultures maintained in total darkness

Callus cultures initiated and maintained in darkness were generally pale-yellow to light brown in color, and produced sufficient masses of friable callus necessary for subculture within three to four weeks. Overall, in the presence of the best hormone regimes (Table 1), 21 to 42% of the callus cultures had 'red/brown-colored' exudates visible either on the callus itself, in the culture medium or in both. The growth rate of the callus cultures declined substantially during the weeks following the initial subculture, resulting in very slow-growing, brown colored clumps of callus (Fig. 1b).

Routinely, pale-yellow-colored callus were subcultured, while callus with red/brown-colored exudates were discarded, because it eventually led to callus death. These pale-yellow-colored, slow-growing callus continued to grow with doubling times ranging from two to four months, of which a majority (>90%) eventually turned brown and died.

The callus cultures that survived the first six months in culture usually continued to exhibit very slow growth for up to two years, irrespective of the frequency at which they were subcultured. However, after this long lag, some of these cultures produced callus that exhibited faster growth (Fig. 1c). During the initial culture phase, some of the brown-colored clumps of callus which exhibited either no growth or very slow growth, occasionally produced 'globules' of callus (Fig. 1d). These callus 'globules' also grew slowly, similar to its parent callus. This phenomenon was most common in *T. cuspidata* and *T.* × *media* cv Densiformis callus cultures maintained in the presence of 5 mg l^{-1} NAA and 0.2 mg l^{-1} kinetin.

Root formation occurred from some of the slow-growing *T.* × *media* cvs. Densiformis and Hicksii, and *T. cuspidata* callus cultures grown on medium containing 2 to 5 mg l^{-1} NAA and 0.2 mg l^{-1} kinetin and maintained in darkness, after more than 18 months in culture (Fig. 1e). The growth of these *in vitro* regenerated roots are very slow and methods to increase their growth rate are presently being evaluated. We have established fast-growing root cultures differentiated from callus cultures of *Cephalotaxus harringtonia*, another gymnosperm that produces compounds exhibiting antineoplastic activity (Wickremesinhe & Arteca 1993b).

Callus cultures maintained under a 16 h light/8 h dark incubation

Callus was also induced from all five explant sources when incubated under a 16 h light/8 h dark day/night regime, in the presence of the same hormone combinations that were successful in the dark. However, the amount of callus

produced and the overall success rate was lower compared to cultures maintained in total darkness (data not shown).

These green-colored callus cultures were more difficult to maintain in culture, compared to the callus cultures initiated in the dark, and eventually died after the first few subcultures. However, we have been able to establish some faster-growing callus cultures of *T.* × *media* cv. Hicksii in the presence of 1.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} kinetin (Fig. 1f). In an attempt to achieve organogenesis, some of these callus cultures were placed on a series of combinations of 2iP and IBA. The formation of shoot primordia-like structures occurred in the presence of 5 mg l^{-1} of both 2iP and IBA; however, we have thus far been unable to produce plantlets. The development of this system and the establishment of these callus cultures are being evaluated.

CR-1 callus cultures

During the first two years, the callus line (CR-1) was subcultured every 6 to 8 weeks. CR-1 has the ability to grow continuously on medium without the presence of exogenously supplied plant growth regulators, and also exhibited faster growth compared to callus cultures established in medium supplemented with 2,4-D and kinetin (Fig. 2).

Media composition and supplements

Supplementing the culture medium with 1.0 g l^{-1} casein hydrolysate significantly increased the growth rate of the CR-1 callus (which was previously grown on B5 basal medium supplemented with 2 × B5 vitamins and 20 g l^{-1} sucrose), while the addition of 0.1 g l^{-1} casein hydrolysate and either 0.1 or 1 mM of both arginine and glutamine had no significant effect on callus growth (Fig. 2), based on mean separations using Scheffe's test ($\alpha = 0.05$). However, none of these supplements had an effect on other callus cultures maintained in the presence of plant growth regulators (Fig. 2). Although cultured cells are normally capable of synthesizing all the required amino acids, the addition of glutamine and other mixtures of amino acids (enzymatic hydrolysates of casein or casamino acids) in the culture medium has been reported (Kadkade 1982; Gamborg 1984). None of the cultures showed any significant differences in the growth rates when placed on medium adjusted to pH values ranging from 4.0 to 7.0.

At this stage, based on the data presented above, the CR-1 callus was routinely cultured on Gamborg's B5 medium supplemented with 2 × B5 vitamins, 20 g l^{-1} sucrose, and 1.0 g l^{-1} of casein hydrolysate.

Sucrose was significantly better than both glucose and fructose for CR-1 callus growth, based on both fresh and dry callus weight (Table 2). Supplementing sucrose (20 g l^{-1}) with both glucose and fructose (2.5 or 10 g l^{-1} each) significantly increased the fresh weight and the dry weight of the callus, compared to sucrose alone (Table 2). Since there were no significant differences in the growth rates between the addition of 2.5 g and 10 g of both glucose and fructose (based on mean separation by the Scheffe's test at the 5 percent level), 2.5 g l^{-1} of both glucose and fructose were routinely used to culture CR-1 callus. There were no significant differences in the dry matter contents (%) within the treatments.

Fig. 1. Callus formation on *T. cuspidata* explants placed on Gamborg's B5 medium supplemented with 20 g l^{-1} sucrose, 5.0 mg l^{-1} α-naphthaleneacetic acid, and 0.2 mg l^{-1} 6-furfurylaminopurine and incubated in darkness for 3 weeks. (*a*) Approximately 6 months old, slow-growing, callus of *T.* × *media* cv. Hicksii cultured in the presence of 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine. (*b*) Approximately 18 months old brown-colored callus culture of *T. brevifolia* cultured in the presence of 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine showing the formation of new callus. (*c*) Formation of callus 'globules' on *T.* × *media* cv Densiformis callus cultured in the presence of 5.0 mg l^{-1} α-naphthaleneacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine. (*d*) Root formation on *T. cuspidata* callus cultured in the presence of 2.0 mg l^{-1} α-naphthaleneacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine. (*e*) Green-colored callus culture of *T.* × *media* cv Hicksii cultured in the presence of 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine and incubated under a 16-h light regime. (*f*) *T.* × *media* cv Hicksii habituated callus (CR-1) placed on membrane rafts and (*g*) cultured in magenta vessels containing Gamborg's B5 medium supplemented with 2 × B5 vitamins, 20 g l^{-1} sucrose, 2.5 g l^{-1} of both fructose and glucose, and 1.0 g l^{-1} of casein hydrolysate (*h*).

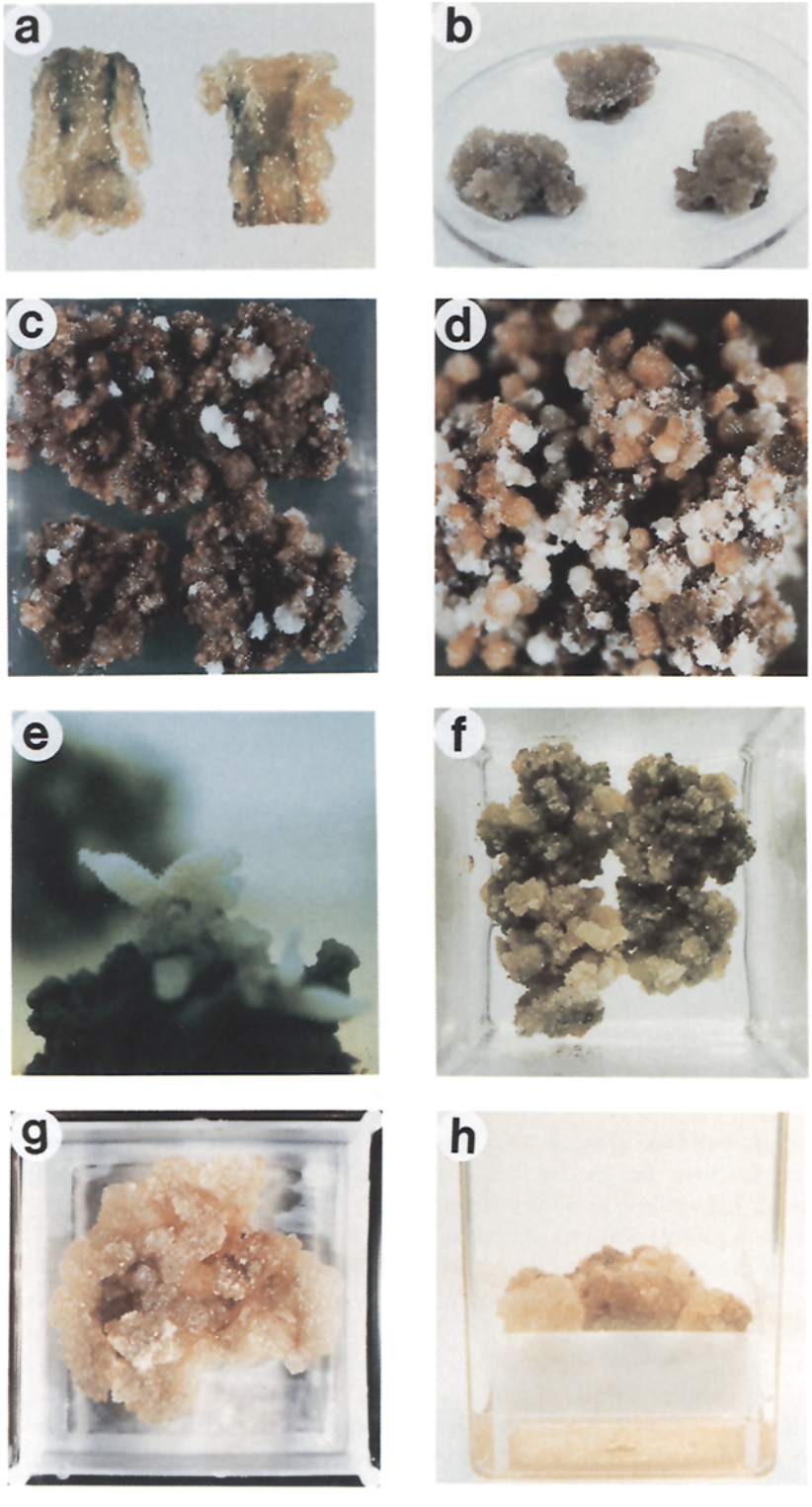

Fig. 1.

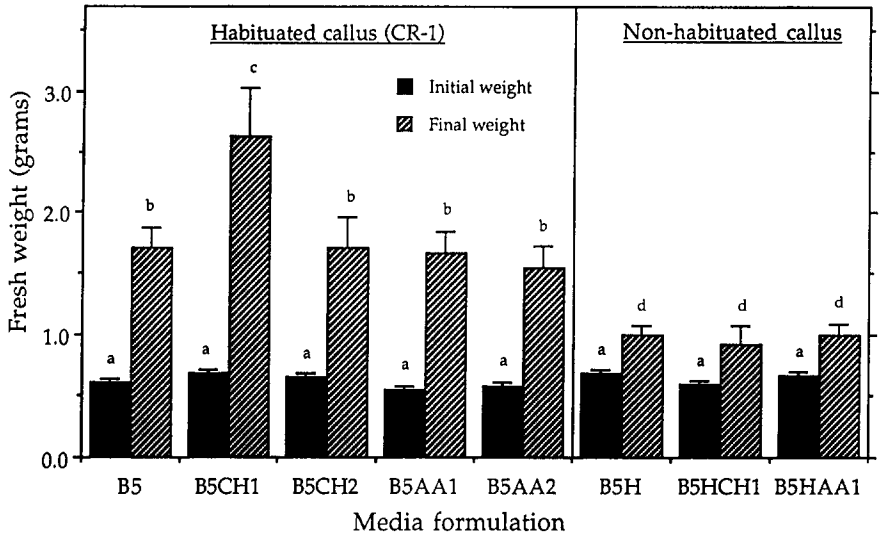

Fig. 2. Effect of casein hydrolysate, and both arginine and glutamine on *Taxus* × *media* cv. Hicksii habituated callus (CR-1) and non-habituated callus. The experiment was conducted in petri plates and data collected after 5 weeks in culture. The weights represent the mean of 10 replications ± standard error. Means separated by Scheffe's test ($\alpha = 0.05$). B5 = Gamborg's B-5 basal medium; CH1 and CH2 = 1.0 and 0.1 mg l^{-1} casein hydrolysate, respectively; AA1 and AA2 = 1.0 and 0.1 mM of both arginine and glutamine, respectively; H = 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg l^{-1} 6-furfurylaminopurine.

Table 2. Effect of type and concentration of sugars on growth of *Taxus* × *media* cv. Hicksii callus. Three grams (fresh weight) of CR-1 callus were placed on magenta GA7 vessels containing Gamborg's B5 salts supplemented with 2 × B5 vitamins, 1.0 g l^{-1} casein hydrolysate, and solidified with 2.0 g l^{-1} gelrite. Each treatment was replicated 5 times and the data was collected after 5 weeks in culture. Suc = sucrose, Glu = glucose, and Fru = fructose.

Sugar (g l^{-1})	Fresh weight (g)[a]	Dry weight (g)[a]
Suc (20)	26.1 ± 1.1 A[b]	0.897 ± 0.010 A[b]
Suc (20) Glu (2.5) Fru (2.5)	28.9 ± 1.7 AB	1.015 ± 0.014 B
Suc (20) Glu (10) Fru (10)	29.2 ± 2.4 B	1.077 ± 0.020 B
Fru (20) Glu (20)	18.7 ± 1.0 C	0.618 ± 0.010 C
Fru (40)	19.5 ± 1.3 C	0.664 ± 0.021 C
Glu (40)	18.9 ± 1.7 C	0.587 ± 0.018 C

[a] Represented as the mean per magenta vessel ± standard error.
[b] Means separated by Scheffe's test ($\alpha = 0.05$).

Preliminary studies indicated that sucrose was better than either fructose or glucose as the carbohydrate source for callus initiation from explants obtained from all five sources. Approximately 90 to 95% of the total explants in culture produced callus within 3 weeks when cultured in the presence of sucrose (at either 20 or 40 g l^{-1}) as described in the materials and methods section. However, similar concentrations of fructose and glucose resulted in a lower percentage of explants exhibiting callus formation (47 to 81%). The long term effect of fructose and glucose on initiation and maintenance of callus cultures was not evaluated.

Both B5 and MS medium were superior to the WP medium for CR-1 callus growth (Fig. 3). The average callus doubling times were 13 and 14 days on the B5 and MS medium, respectively. These doubling times are 2 to 5 times shorter than other callus cultures we have established from both *T.* × *media* and other *Taxus* species (eg. average doubling time for callus line LG1-1 is 24 days). These doubling times were calculated based on the fresh weight growth data, because it was not feasible to obtain dry weights for each data point represented in the growth curves (Fig. 3). However, dry weights were obtained for CR-1 callus cultured on B5 medium, where mem-

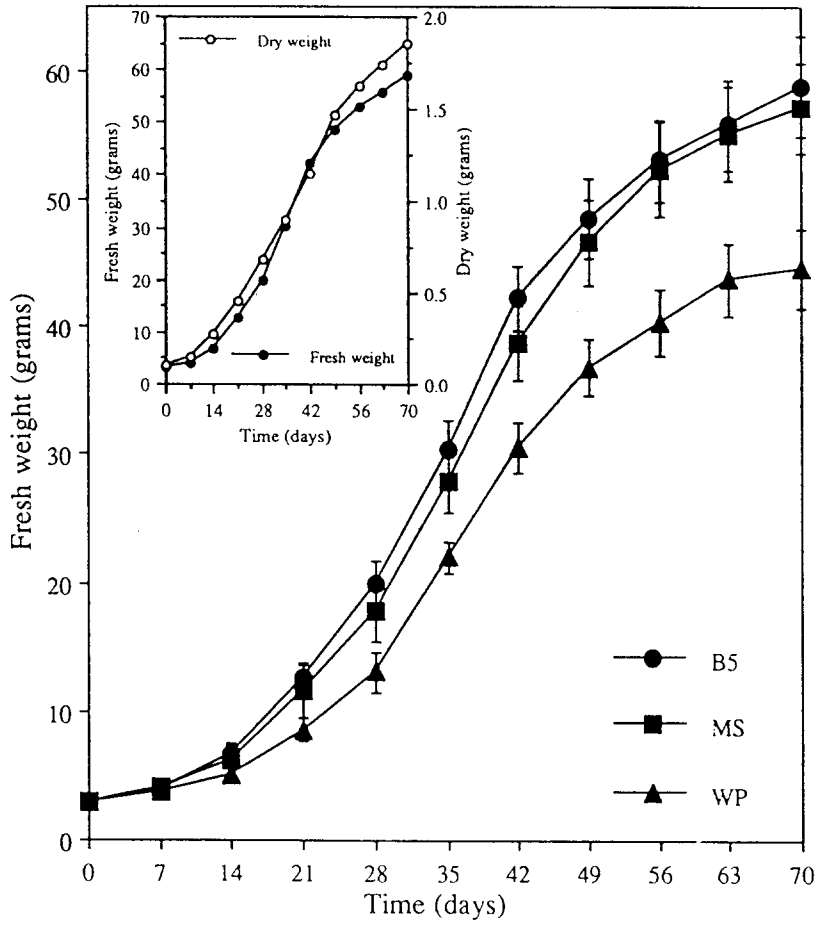

Fig. 3. Growth curves for *Taxus* × *media* cv. Hicksii callus (CR-1) cultured on Gamborg's B5 medium (B5), Murashige and Skoog medium (MS), and McCown's woody plant medium (WP) supplemented with 2 × B5 vitamins, 20 g l^{-1} sucrose, 2.5 g l^{-1} each of glucose and fructose, and 1.0 g l^{-1} casein hydrolysate. Initially three grams of callus were placed per membrane raft, and the rafts aseptically weighed to obtain the fresh callus weight. Each data point represents the mean of five rafts ± standard error. Inset: growth curves for same callus cultured on B5 medium, based on fresh and dry weight.

brane rafts were randomly harvested each week for taxol analysis. The increase in fresh weight is well correlated to the increase in dry weights (Fig. 3, inset).

The B5 medium was selected over the MS medium for all subsequent studies with CR-1 callus, although they both achieved similar results. The major difference between these two media formulations (based on the macronutrient composition, especially the nitrogen) is that the MS medium contains 40 mM nitrate and 20 mM ammonium, compared to 25 mM nitrate and 2 mM ammonium in the B5 medium. In contrast, the WP medium contained 10 mM nitrate and 5 mM ammonium.

The use of membrane rafts facilitated the culture of CR-1 callus longer without the need of subculture, since the culture medium could be changed without disturbing the callus (Fig. 1g and 1h). There were no significant differences in the growth rates when callus grown on membrane rafts were compared to callus grown on 100 ml of solidified media in Magenta GA7 vessels. However, the callus could be maintained only 7 to 8 weeks without subculture, compared with 10 to 12 weeks on membrane rafts.

Characterization of CR-1 callus

The CR-1 callus had a pale yellow color up to about the sixth week following the subculture onto membrane rafts and then gradually changed to a light brown color. By the 9th week, a majority of the callus was brown, and by the 12th week, all the callus were dark brown. The pale-yellow-colored callus (6 to 9 weeks old) could be successfully subcultured; however, cultures that were older than 10 weeks and dark brown did not generally contain callus which could be subcultured.

The mitotic index of the pale-yellow-colored callus during the first six weeks of culture ranged from 2 to 3%, indicating active cell division, which also coincided with the fastest growing phase of the callus cultures. The mitotic index gradually decreased after six weeks in culture, which coincided with the stage during which the color of the callus changed gradually from pale yellow to brown. There were no mitotic figures observed in the dark brown cells (older than 10 weeks) out of a total number of more than 500 cells scored, suggesting that the callus was actively dividing and growing during the first six weeks following the subculture of callus on to membrane rafts and that mitotic activity decreased during the subsequent weeks in culture.

Most of the brown areas originated on the peripheral areas of the callus, while the callus in close contact with the nutrient medium were still pale yellow. These pale yellow cells exhibited a mitotic index ranging from 0.2 to 0.8%. Caplin (1947) demonstrated that the most rapid growth of callus occurs at the points where there is contact between the cells and the growth medium, while as growth progresses, its rate varies in different areas within the callus.

Taxol acts on cellular and spindle microtubules and disrupts cell division (Horowitz et al. 1986). In general, antitumor compounds exhibit some cytotoxicity to any living cell, and is more pronounced in actively dividing cells. Cultured cells are usually rapidly dividing and proliferating, compared with somatic cells of intact plants. However, the exact role (if any) taxol plays in *Taxus* cells – both *in vivo* and *in vitro* – as well as how it is compartmentalized and metabolized inside the cell is unknown.

Preliminary studies on visualizing the microtubules in *Taxus* root-tip cells using antibodies against tubulin indicated that the microtubules in these cells were much thicker (probably bundled) and fewer in number (compared to onion root-tip cells).

Analysis of taxol in CR-1 callus and other callus cultures

The HPLC protocol gave good separation of taxol from crude callus extracts (Fig. 4). A scatter diagram depicting the levels of taxol produced by the CR-1 callus over a period of 14 weeks following subculture onto membrane rafts, is given in Fig. 5. The highest value obtained from CR-1 callus represented a taxol yield of 13.1 mg kg^{-1} (0.0131%), expressed on a dry callus weight basis. Each data point corresponds to a representative sample of callus

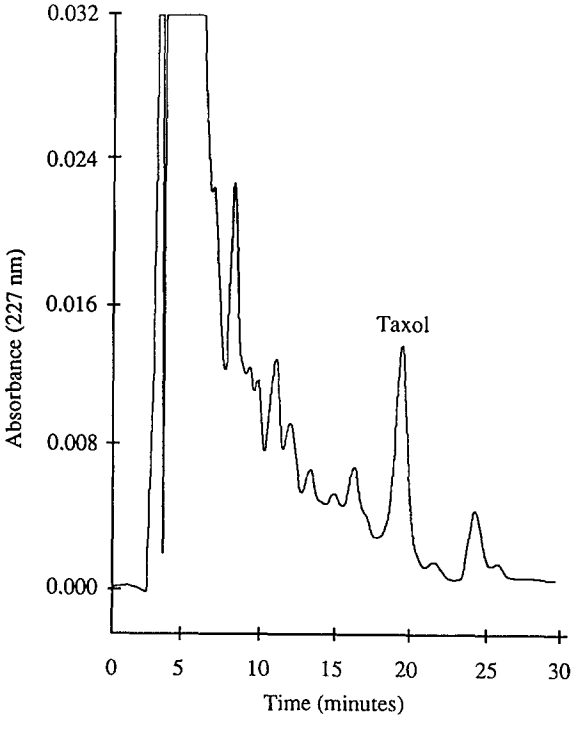

Fig. 4. HPLC separation of taxol from a *Taxus* × *media* cv. Hicksii callus (CR-1) extract. Analysis was performed on a Dynamax 60 Å 8 μm phenyl column (4.6 mm × 250 mm) using a mobile phase consisting of methanol:50 mM acetate buffer (pH = 4.4):acetonitrile (20:41:39), at a flow rate of 1 ml per min, and absorbance detected at 227 nm.

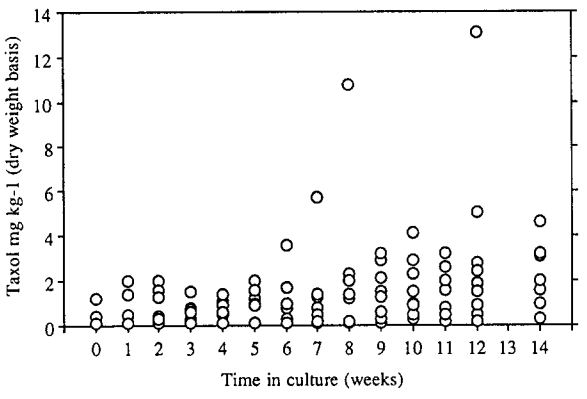

Fig. 5. Scatter diagram depicting the variation in taxol levels observed in the *Taxus* × *media* cv. Hicksii callus (CR-1) cultured on membrane rafts. Gamborg's B5 medium supplemented with 2 × B5 vitamins, 20 g l^{-1} sucrose, 2.5 g l^{-1} of both fructose and glucose, and 1.0 g l^{-1} of casein hydrolysate was used as the culture medium. Each week an entire membrane raft was harvested and a representative sample of that callus was used for analysis. Data from at least five membrane rafts are represented for each weekly time point.

cultured on a single membrane raft. Data for each week is represented by at least 5 replications. This data represent samples analyzed over a two year period; at which stage the CR-1 callus itself was 4 to 5 years old. The data clearly displays the tremendous heterogeneity among and within the callus with respect to the amounts of taxol produced.

Regression analysis of this data (represented by 112 samples) exhibited an increasing trend ($R^2 = 0.151$) in taxol production in relation to the culture age, suggesting that the older, brown-colored callus contain higher levels of taxol compared to the younger pale-yellow-colored callus. As mentioned earlier, the pale-yellow younger callus contained the most mitotically active cells, while the brown callus contained more quiescent cells (non-dividing). These observations confirm the fact that although callus cultures provide a morphologically more uniform material than the whole plant, there is heterogeneity between and within callus. The overall age of the callus did not seem to determine the taxol productivity.

It has been suggested that there is an inverse relationship between the growth rate and the rate of secondary metabolite accumulation (Misawa & Endo 1988). Therefore, routine selection for fragile, faster growing callus could have resulted in the selection of cells that produce lower amounts of secondary metabolites.

The amounts of taxol produced in the other callus lines we have established are shown in Table 3. The levels of taxol found in these callus lines range from 1.7 to 14.2 mg kg^{-1} (0.00017 to 0.00142%) on a dry weight basis. All these callus cultures are slow-growing compared to CR-1 callus, exhibit doubling times ranging from 3 to 6 weeks, and have been in culture for more than 18 months. Callus line LG1-1 has been maintained under a 16-h light regime, while all other lines are dark-grown cultures. Studies are in progress to improve the growth rates of these callus lines.

Confirmation of taxol in CR-1 callus

Taxol was identified by HPLC techniques described in the methods section. Approximately two milligrams of taxol was purified from CR-1 callus collected over a period of time and bulked together, as described by Wickremesinhe and Arteca (1993a). Taxol purified from callus exhibited microtubule stabilizing activity similar to authentic taxol. Microtubule assembly occurred

Table 3. Taxol content in different *Taxus* callus lines. The values represent the mean ± standard error expressed on a dry matter basis, based on 6 to 12 samples per callus line. All callus samples used for analysis were in culture for 18 months or more.

Taxus source	Taxol (mg kg^{-1})
brevifolia	
PB-A	7.0 ± 2.5
GB-1	5.4 ± 2.2
baccata cv. Repandens	
PR-A	2.4 ± 1.4
×*media* cv. Densiformis	
PD-A	10.0 ± 3.9
cuspidata	
PC-A	14.2 ± 2.4
GTT-1	8.3 ± 2.3
×*media* cv. Hicksii	
PH-A	9.2 ± 3.0
G2-1	10.6 ± 5.0
CRG-1	5.7 ± 1.8
LG1-1	1.7 ± 0.6

in the absence of exogenously added GTP, and the assembled microtubules did not depolymerize when exposed to cold (4°C), which is characteristic of taxol action. Controls without taxol did not show any microtubules.

The one-dimensional ^{1}H spectrum and the two-dimensional $^{1}H-^{13}C$ inverse NMR detected spectrum verified that taxol was produced by CR-1 callus cultures. The chemical shifts and the line-shapes observed for this product were identical to those observed for authentic taxol reported by Falzone et al. (1992), thus confirming the production of taxol in CR-1 callus.

Conclusions

We have demonstrated the ability to induce callus from five explant sources derived from four *Taxus* species. All these callus cultures are initially very slow-growing. However, after long periods in culture, some callus cultures have the ability to grow much faster than the initial parent callus. Although callus was generated from all five explant sources, the cultures established from *T.* × *media* were the fastest growing and the most amenable to tissue culture, while the callus from *T. baccata* were the most difficult to maintain in culture.

The availability of fast-growing cell lines is a key factor in scale-up cell culture operations. A major objective of this study was to optimize cell growth on defined media formulations, thereby establishing sustained growth of callus cultures over long periods of time.

This report demonstrates (a) three lines of evidence for the production of taxol in tissue cultures: taxol was detected in crude callus extracts using conventional reverse phase HPLC techniques, taxol was isolated in milligram quantities from callus cultures, its biological activity was demonstrated with the microtubule-stabilizing bioassay, and its structure was confirmed by NMR spectroscopy, and (b) the production of taxol in callus cultures we maintained for over 5 years in culture.

Studies on establishment of cell suspension cultures, the use of elicitors, and adaptation of cells for scale-up culture in bioreactors are in progress.

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