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Production of taxoids with biological activity by plants and callus culture from selected *Taxus* genotypes

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Abstract

Twenty seven different yew trees belonging to various genotypes and hybrids have been screened for their capacity to produce significant amounts of taxoids provided with biological activity in the tubulin test. From the three best genotypes selected, *Taxus* x *media* "Sargentii" proved to be able to produce viable calluses from excised roots placed in vitro. Taxoid composition at various times of the in vitro culture was determined and the carcinostatic efficiency of the extracts was established using the KB cell cytotoxicity test. In leaves and calluses, respectively, 0.069 and 0.032% paclitaxel (taxol) contents were found. These contents were significantly higher than those previously reported for other genotypes.

Keywords: Biological activity; 10-Deacetylbaccatin III; In vitro culture; Paclitaxel; Taxoids; Taxol; *Taxus*

1. Introduction

Paclitaxel (taxol) and its semi-synthetic derivative docetaxel (taxotere) are two important alkaloids of the taxane series used as anticancer drugs. Paclitaxel was initially obtained from the Pacific yew tree *Taxus brevifolia* (Wani et al., 1971). Their carcinostatic property lies in their capacity to inhibit cell proliferation by promoting the assembly of tubulin into microtubules that are stabilized against depolymerisation (Horowitz et al., 1986). These compounds can also be obtained by semi-synthesis from readily available 10-deacetylbaccatin III (10-DAB) isolated from yew needles (Denis et al., 1988). Due to the relatively low yield (0.01 to 0.06% of the dry wt) of both, the interesting molecules and their precursors when extracted from trees (El Sohly et al., 1995) and to the poor growth rates of yews, various attempts to produce taxoids through callus or cell cultures have been developed as potential alternative sources. Taxol was obtained from *Taxus cuspidata* callus culture (Fett-Neto et al., 1992, 1993). *Taxus brevifolia* culture produced no taxol but only 7-xylosyl-10-deacetyl taxol (Gibson et al., 1993). In liquid cell culture of *Taxus chinensis*, a range of 16 taxoids including traces of 10-DAB and paclitaxel were isolated, as well as five novel compounds that were not present in the plants (Mehnard et al., 1998). Traces of 10-DAB were also found in callus culture of *Taxus baccata stricta* (Zhiri et al., 1995). Taxol was also found to be produced by cell cultures in liquid medium of *Taxus canadensis* and *Taxus cuspidata* but the production became highly variable after successive subcultures (Ketchum and Gibson, 1996).

In view of improving the capacity of yew cell cultures to synthesize taxoids, we have screened a range of yew species and hybrids on the basis of both the amount of taxoid and taxoid precursors (10-DAB and baccatin III) in crude leaf extracts using HPLC appropriate columns (Ketchum and Gibson, 1993) in order to select the best producers. Two biological assays, the tubulin test (Shelanski et al., 1973) and the KB test of toxicity (Borenfreund and Puerner, 1985) were also applied for each extract. For three of the selected genotypes, in vitro culture was established from sterile plantlets micropropagated in vitro. Both plants and callus culture of

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the genotype *Taxus* x *media* "Sargentii" were found to synthesize significant levels of taxoids provided with biological activity, that could be interesting as a novel source of natural yew products.

2. Results and discussion

2.1. Preliminary tests of selection of yew genotypes

The list of individual yews studied as well as the yields of extraction, their content of taxoid and their efficiency in the tubulin test are shown in Table 1. A preliminary selection was conducted on the basis of both the amount of 10-DAB and baccatin III contained in leaves and the biological activity of the extracts using the tubulin test. With the exception of "Summergold" cultivar (No. 22), *Taxus baccata* extracts showed only a weak activity. In contrast, the extracts from four hybrids displayed an activity that was of interest, namely *T.* x *media* "Sargentii" (No. 27), *Taxus* x *media* "Hillii" (No. 26), *Taxus* x *media* "Hatfieldii" (No. 24) and root extracts of *Taxus* x *media* "Hicksii" [No. 25(2)]. One can observe relatively high contents of 10-DAB exceeding 1% in the extracts, while the percentage of baccatin III present is much lower except in *T.* x *media* "Sargentii" (No. 27) and *T.* x *media* "Hicksii" [25(1) and 25(2)] with respective contents of 0.22, 0.43 (leaves) and 0.20% (roots).

2.2. In vitro culture and callus taxoid contents

Leaf, stem and root explants from the three hybrids selected (Nos. 22, 24, 27) for their content in 10-DAB and baccatin III and for their efficiency in the tubulin test were grown in vitro to check their ability to produce calluses. The root explants from *T.* x *media* "Sargentii" produced pale stained and fast growing calluses that developed actively from the 18th day on. These calluses were maintained over a 3-year period through regular subculturing. In contrast, explants from the other genotypes also start dividing within 3 weeks but gave rise to small and slow growing calluses that turned brown rapidly and stopped growing after one month. Consequently, only the *T.* x *media* "Sargentii" calluses were retained to check their taxoid composition and for the subsequent biological tests.

Table 1
Extraction yields, 10-DAB and baccatin III (bac III) contents and tubulin test for 27 yew species and hybrids

No.	Yews and cultivars	Extraction yields (% w/w)	10-DAB extract (% w/w)	10-DAB leaves (% w/w)	Bac III extract (% w/w)	Bac III leaves (% w/w)	Tubulin test IC_{50}(mg/ml)
1	*T. baccata* (F)	3.50					>10
2	*T. baccata* (F)	6.60					>10
3	*T. baccata* (F)	8.60					>10
4	*T. baccata* (M)	4.86					>10
5	*T. baccata* (M)	6.60	0.73	0.048	0.031	0.0020	6.35
6	*T. baccata* (M)	6.10	0.51	0.031	0.053	0.0032	8.59
7	*T. baccata* (M)	4.90	1.09	0.053	0.054	0.0026	5.38
8	*T. baccata* "Adpressa" (F)	4.40	1.05	0.046	ndl	ndl	3.41
9	*T. baccata* "Adpressa Aurea" (M)	4.65					>10
10	*T. baccata* "Adpressa Aurea" (M)	3.74					>10
11	*T. baccata* "Argenteo marginata" (M)	5.25	0.18	0.010	0.012	0.0006	2.82
12	*T. baccata* "Corona" (M)	4.34	0.38	0.016	0.098	0.0043	4.83
13	*T. baccata* "Corona" (M)	5.36	0.58	0.031	0.065	0.0035	3.25
14	*T. baccata* "Dovastonii" (F)	5.60	0.62	0.035	0.026	0.0015	3.82
15	*T. baccata* Elegantissima (M)	4.33					>10
16	*T. baccata* "Erecta" (F)	5.31	0.71	0.038	0.062	0.0033	2.48
17	*T. baccata* "Fastigiata" (M)	5.18	0.97	0.051	0.047	0.0024	2.75
18	*T. baccata* "Fastigiata" (F)	7.12	0.85	0.061	0.055	0.0039	2.80
19	*T. baccata* "Fructuluteo" (F)	18.20	0.11	0.019	0.008	0.0015	>10
20	*T. baccata* "Overeynderii" (M)	5.60	1.03	0.058	0.105	0.0059	4.33
21	*T. baccata* "Semperaurea" (F)	3.86	0.83	0.032	0.034	0.0013	3.08
22	*T. baccata* "Summergold" (F)	5.06	0.27	0.014	0.047	0.0024	0.31
23	*T. brevifolia* (M)	5.42					>10
24	*T.* x *media "Hatfieldii"* (M)	5.35	0.13	0.007	0.055	0.0029	0.15
25(1)	*T.* x *media* "Hicksii" (leaves) (F)	2.42	1.08	0.026	0.428	0.0104	4.41
25(2)	*T.* x *media* "Hicksii" (roots) (F)	0.85	0.24	0.002	0.202	0.0017	1.00
26	*T.* x *media* "Hillii" (M)	5.35	0.20	0.011	0.074	0.0039	1.09
27	*T.* x *media* "Sargentii" (M)	4.44	0.53	0.024	0.218	0.0097	0.62

M: male, F: female. ndl: No detectable level. IC_{50} paclitaxel: 0.006 mg/ml. The genotypes giving a IC_{50} >10 in the tubulin test have not been analysed for their 10-DAB and baccatin III contents.

Callus samples from several boxes (5 for each analysis) were pooled at different stages of culture from 70 to 127 days and were extracted by solid phase extraction (SPE) according to the procedure described by van Rozendaal et al. (1997, 2000) and used at a semi preparative scale. The taxoids were quantitatively recovered from the methyl *t*-butyl ether fractions. The chromatogram profiles of the extract containing taxoids are presented on Fig. 1a–c and compared with that of standard 10-DAB, baccatin III, cephalomannine and paclitaxel (Fig. 1d). None of the fractions contained detectable levels of 10-DAB. Small amounts of baccatin III and cephalomannine were present. On the other hand, paclitaxel represented one of the most important peaks (peak 2). The chromatograms showed the occurrence of other abundant unidentified taxoids detectable by diode array detector [Fig. 1e (peaks 1, 3, 4, 5)]. Comparison of the three-chromatogram profiles showed that the relative proportion of taxoids varied according to the age of the callus, the 96-day-old callus showing

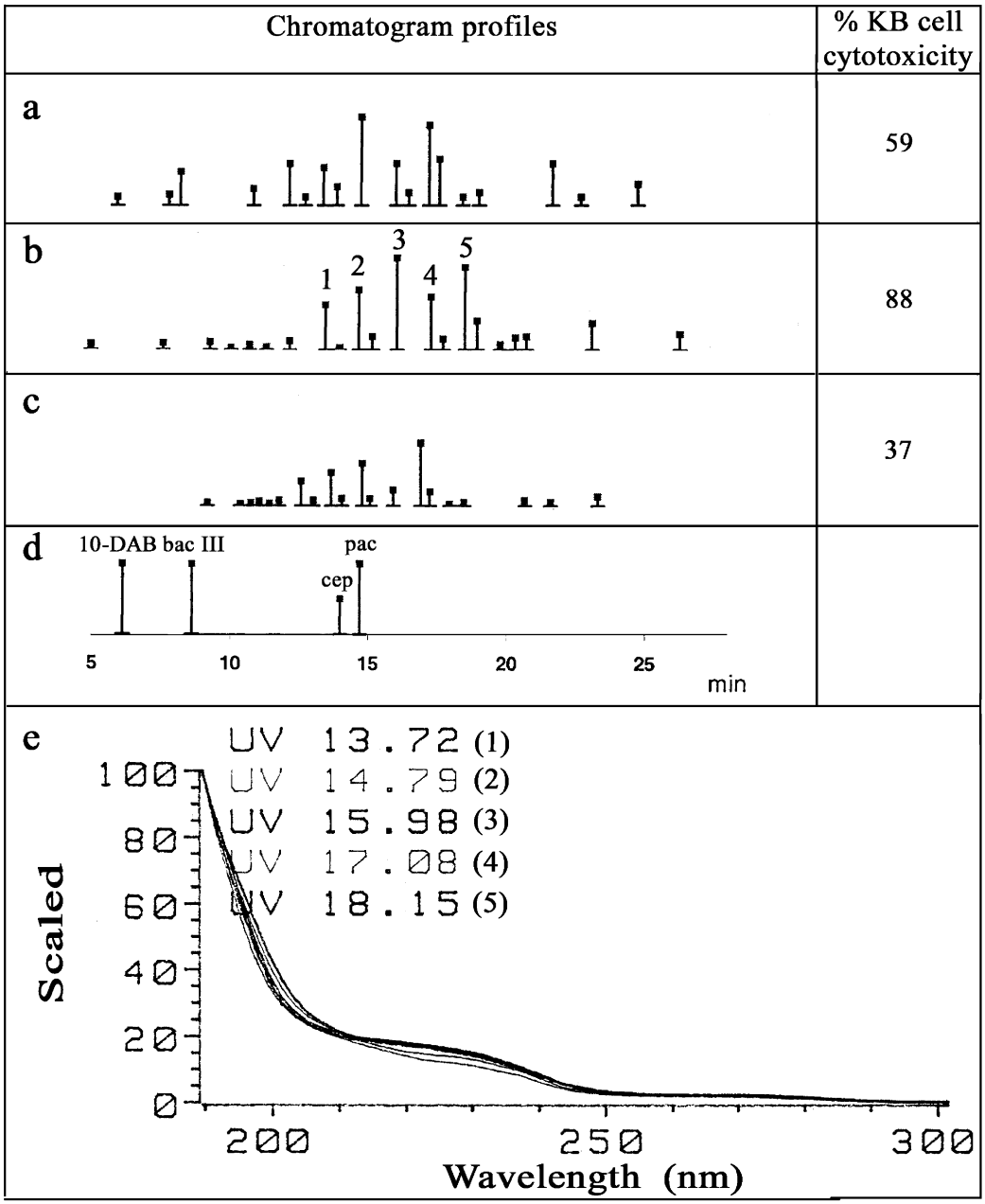

Fig. 1. The chromatogram profiles are presented with the corresponding percentages of dead cells in the KB cell cytotoxicity test. Taxoid composition in 70- (a), 96- (b) and 127- (c) day-old *T.* x *media* "Sargentii" calluses. (d) Standards (10-DAB, Rt: 6.1 min; baccatin III (bac III), Rt: 8.6 min; 3: cephalomannine (cep), Rt: 14.0 min; paclitaxel (pac), Rt: 14.8 min). (e) Diode array scans over the 200–300 nm range of the absorption spectrum of peaks 1–5 from chromatogram b.

the largest range of taxoids. It was also found that the 18.2 min taxoid peak was only present in the 96-day-old extract. This suggests that taxoid secondary metabolite accumulation is the result of a dynamic equilibrium, intermediate compounds undergoing biotransformations. As reported by Menhard et al. (1998), the production of taxoids seems to be growth-linked.

From a quantitative aspect, the paclitaxel content of the 96-day-old callus was 0.032% dry wt whereas the paclitaxel content of the mother tree was 0.069% (dry wt). Paclitaxel amount in the callus is nearly half of that of the mother tree leaves of *Taxus* x *media* "Sargentii" but is substantially higher than any result previously reported from the calluses of a large number of *Taxus* genotypes: *Taxus cuspidata* (0.02%) and *Taxus canadensis* (Fett-Netto et al., 1992, 1993), *Taxus brevifolia* (none) (Gibson et al., 1993), *Taxus* x *media* "Hicksii" and other genotypes (0.013%) (Wickremesinhe and Arteca, 1993), *Taxus baccata* (none) (Vanek et al., 1996), *Taxus wallichiana* (none) (Banerjee et al., 1996), *Taxus* x *media* "Hatfieldii" (0.009%) (Furmanowa et al., 1997), *Taxus chinensis* (trace) (Menhard et al., 1998). This strain of yew cells appears to be a good starting point for further optimisation through the use of elicitors.

The presence of cytotoxic taxoids in the extracts was confirmed by the KB test (Fig. 1) with cytotoxic effects particularly high for the 96-day-old callus extract. The question rises now to determine whether the high cytotoxicity found in the extract is only due to the presence of paclitaxel or whether some other taxoids participitated in this cytotoxic activity.

3. Experimental

3.1. Plant material and in vitro culture

3.1.1. Plant material

Most of the *Taxus* genotypes or hybrids used were harvested in the National Arboretum of Chèvreloup-France (Table 1). One (No. 7) was harvested at the C.N.R.S. station of Gif-sur-Yvette (France) and the last one (No. 25) was supplied by a nursery (Briant S.A., Angers, France). Their nature (male or female) was determined and samples for biochemical analysis were all collected in February to avoid possible seasonal variations (Vance et al., 1994; Hook et al., 1999).

3.1.2. In vitro culture

Genotypes Nos. 22, 24 and 27 were micropropagated through in vitro culture in order to provide sterile material for callus establishment. For micropropagation, microcuttings were excised from juvenile 1-year-old shoots and disinfected with 70% alcohol, then with 8% calcium hypochlorite for 20 min and washed in sterile water. They were placed on Murashige and Skoog (1962) medium modified according to Zhiri et al. (1994) and containing various naphthalene acetic acid (NAA) and benzyladenine (BA) concentrations. Medium was adjusted to pH 5.8 ± 0.1 with 1 N KOH before autoclaving for 20 min at 120 °C. In vitro cultures were maintained at 24 °C under a 16 h photoperiod. The photon fluence rate was 70 μmol m^{-2} s^{-1} from daylight Osram 25 W tubes (Durolux, Courbevoie, France).

For callus establishment, leaf, stem and root explants from the sterile micropropagated plantlets were placed on solid Gamborg B5 medium (Gamborg et al., 1968) modified according to Fett-Neto et al. (1992) and containing 0.5 mg/l NAA, 0.2 mg/l kinetin, 30 g/l sucrose, 1 g/l casein hydrolysate (Sigma, L'Isle d'Abeau Chesnes, France), 100 mg/l ascorbic acid, 10% (v/v) cocomilk (Sigma) and 0.8% agar. pH was adjusted to 5.8 ± 0.1 before autoclaving. Callus cultures were grown into Magenta boxes (Sigma) under darkness in growth chamber at 24 ± 1 °C and subcultured every 6–8 weeks.

3.2. Extraction

3.2.1. From the plant material

Dried needles (50–100 g) were powdered prior to extraction and percolated with methanol (5×200 ml for 20 min), filtered and evaporated to dryness in vacuo. The residue was partitioned between water (100 ml) and dichloromethane. The dichloromethane extract was dried over anhydrous sodium sulfate, evaporated and analysed by TLC. The precoated plates (silicagel 60 F 254, Merck) were developed in dichloromethane/methanol 95/5 (v/v). Paclitaxel was detected by UV at 254 nm, then by spraying a 3% sulphuric acid methanolic solution followed by heating at 115 °C for 5 min (visualization UV at 365 nm).

3.2.2. From callus samples

Callus samples were freeze-dried, extracted by methanol and the extraction yields were determined. Solid phase extractions were performed on pretreated ChemElut cartridges (Varian-France) with 10 ml of 0.5% sulphuric acid to retain taxin alkaloids. A 200 mg extract sample was applied at the top of the cartridge in a minimum volume of methanol. After 30 min, the extract was successively eluted by 60 ml petroleum ether, by 140 ml of a mixture of petroleum ether/methyl *t*-butyl ether 80/20 (v/v) and finally by 200 ml of methyl *t*-butyl ether where taxoids are concentrated. The solvent of this last fraction was evaporated under reduced pressure and the residue was weighed.

3.3. High-performance liquid chromatography (HPLC) of taxoids

The HPLC system consisted of a Beckman 126 pump and a 1040 A Hewlett-Packard photodiode array detector.

Separations were performed on a Phenomenex Curosil G column (4 μm; 250×4.6 mm) preceded by a Curosil G guard column (30×4.6 mm) using acetonitrile-water gradient as mobile phase at a flow rate of 0.8 ml/min. Injections were performed via a 7125 Rheodyne sample injector fitted with a 20-μl loop. The water-acetonitrile elution profile started at 40% acetonitrile and was linearly increased to 75% acetonitrile over 20 min and was followed by an isocratic step for 10 min. Peaks were recorded over the 190–300 nm range of the absorption spectrum and the chromatograms were plotted at 227 nm. Taxoid standards were supplied by the ICSN (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France). Paclitaxel contents in tree and callus extracts were determined according to the same procedure as described above by reference to a calibration curve prepared with dilutions of pure paclitaxel.

3.4. Biological tests

3.4.1. Tubulin test

The tubulin test was performed as previously described (Lataste et al., 1984). Each extract was dissolved in DMSO to a concentration of 10 mg/ml. One microliter of this solution was added to 150 μl of the tubulin preparation at 37 °C. The mixture was then placed in a temperature-controlled UV cell at 4 °C. Antitubulin activity was determined by monitoring the temperature-dependent in vitro assembly of microtubules by turbidimetry at 350 nm. Tests were performed comparatively to a paclitaxel standard, on the same day at the same conditions.

3.4.2. KB test

Cytotoxicity was evaluated on microwell plates covered with a 24 h confluent layer of KB cells. The KB cells were incubated during 72 h in the presence of 10 μg ml^{-1} of the taxoid fraction isolated from the calluses. Successive dilutions of callus fractions were displayed on the microwell plates. The cytotoxicity was determined by photometric measurement of the staining of viable cells after neutral red absorption (Borenfreund and Puerner, 1985).

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