Variation of Taxane Content in Needles of *Taxus* x *media* Cultivars with Different Growth Characteristics

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Needles from 17 different *Taxus* x *media* cultivars, belonging to 4 groups showing different growth characteristics, were analyzed using high performance liquid chromatography for their content of 10-deacetylbaccatin III, baccatin III, cephalomannine and paclitaxel (Taxol®). The 4 *Taxus* x *media* cultivar groups were: 1.) medium to fast growing and upright form; 2.) slow growing and upright form; 3.) fast growing and spreading form; and 4.) slow growing and spreading form. The purpose of this study was to identify yew cultivars of fast growth rate, upright growth and high taxane content in their needles. The highest content of paclitaxel was found in 'Coleana' of group 1 (378 μg/g of the extracted dry weight). Three cultivars in group 1, 'Coleana', 'Stovekenii' and 'Hicksii', make good candidates for taxane extraction because of their high paclitaxel and 10-deacetylbaccatin III content, fast biomass accumulation and upright growing form. They are also good starting materials to develop alternative methods for the production of paclitaxel and its analogous compounds through modern biotechnology approaches.

Key words: HPLC, Paclitaxel, Taxane, *Taxus* x *media*

Introduction

Paclitaxel (Taxol®), showing excellent antitumor activity against breast cancer, lung cancer, head and neck cancer, is one of the best chemotherapeutic agents developed from plant sources (Arbuck and Blaylock, 1995). Its specific anticancer activity stems from the fact that it promotes microtubule formation and inhibits post-mitotic spindle disassembly (Schiff *et al.*, 1979). This compound was first isolated from the bark of Pacific yew (*Taxus brevifolia*) in 1971 (Wani *et al.*, 1971). Since the content of paclitaxel in this original source is very low, various research efforts have been tried to solve its supply problem. Among them, total synthesis of paclitaxel was achieved but is far from being commercially cost-efficient (Borman, 1994). Currently a large portion of the paclitaxel in the market comes from semi-synthesis, in which paclitaxel is synthesized from its relatively abundant precursors, such as 10-deacetylbaccatin III (10-DAB) (Kingston *et al.*, 1990). Since high-taxane-yield *Taxus* species and cultivars are crucial for the extraction of paclitaxel and 10-DAB, many different *Taxus* species and cultivars were screened (Vidensek *et al.*, 1990; Elsohly *et al.*, 1995; van Rozendaal *et al.*, 2000; Parc *et al.*, 2002).

In addition to the variable taxane content, different species and cultivars have different growth characteristics. Fast-growing yew trees accumulate a larger amount of biomass in their renewable parts (like needles) in a given period of time than those of a slow growth rate. It was reported that needles contain taxane with an amount comparable to that in its original source (Witherup *et al.*, 1990). Therefore, it is desirable to identify some cultivars which can combine both high taxane content and fast biomass production. Besides growth rate, other characteristics like upright or spreading growing form are also important for clippings collection, since upright growing shoots make me-

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chanical collection easier. In this report we collected needle samples from 17 *Taxus* x *media* cultivars with different growth characteristics. The content of 4 important taxanes (10-DAB, baccatin III, cephalomannine and paclitaxel) was then analyzed by high performance liquid chromatography (HPLC). In this study we identified several *Taxus* x *media* upright growing cultivars with both high taxane content and fast accumulation of biomass. These cultivars make good candidates for direct taxane extraction from clippings, meanwhile, they are also good starting materials for the alternative production of paclitaxel through biotechnological approaches, including genetic improvement and metabolic engineering.

Materials and Methods

Taxane standards

Paclitaxel standard (>95% purity) was provided by the National Cancer Institute (NCI), Bethesda, MD, USA. Standards of 10-DAB, baccatin III and cephalomannine were donated by Dr. David G. I. Kingston of the Chemistry Department, Virginia Polytechnic Institute and State University, USA.

Plant materials

Twigs were collected from the Secrest Arboretum, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA. Arboretum accession numbers are available upon request. Sample of each cultivar included bulked twigs from two to three trees. Right after twig collection in February 2001, fresh needles were removed from the branches for taxane content analysis by HPLC.

Taxane extraction

10 g of fresh needles were first chopped with a scalpel. The chopped needles were then mixed with glass beads (425–600 microns; Sigma) and ground in a mortar. The ground tissues were soaked in 45 ml of hexane with occasional shaking to remove nonpolar components like wax. After 18 h, the hexane solution was carefully discarded and the remaining hexane in the extracted plant material was subject to evaporation overnight at room temperature. Then 100 ml of MeOH were added to extract taxane by constant stirring at room temp. in the dark for 18 h. The resulting MeOH extraction solution was then filtered through a glass filter and the needle tissue residue was dried until constant weight at 45 °C to allow determination of extracted dry weight (EDW). The MeOH extract residue was re-dissolved in 15 ml MeOH. This MeOH solution was then subjected to pre-HPLC treatment using a solid phase extraction (SPE) cartridge to remove part of the interfering non-taxane compounds.

Solid phase extraction (SPE) purification

An aliquot of 1.5 ml of the methanol extract from the above step was brought to 5 ml by H_2O and subjected to SPE. The Supelco LC-18 SPE cartridge (Supelco, Bellefonte, PA, USA) was first conditioned with 2 ml of MeOH followed by 2 ml of H_2O. After loading 5 ml of the diluted MeOH extract, the column was washed with 2 ml of H_2O twice, followed by 2 ml of 20% MeOH (in H_2O, v/v). The taxanes were then eluted with 2 ml of MeOH from the column and the eluate was dried in a speed vacuum. The residue was then re-dissolved in 200 μl MeCN and 10 μl of this sample were applied to the HPLC column.

High performance liquid chromatography (HPLC)

The taxane HPLC analyses were performed on a Novapak Phenyl column (4 μm, 60 Å, 150 × 3.9 mm) from Waters Corporation (Milford, MA, USA). The mobile phase was composed of two solvents: A, 0.05 M NH_4OAc/MeCN (7:3, v/v), and B, 0.05 M NH_4OAc/MeCN (1:9, v/v). For quantitation of 10-DAB, baccatin III and cephalomannine, the gradient elution program of Theodoridis *et al.* (1998) was followed. For paclitaxel analysis, a modified program (Table I) was used for improved separation. The flow rate of the mobile phase was 0.8 ml/min. The injection volume was 10 μl. All peaks were detected at 227 nm. The R_t for 10-DAB, baccatin III, cephalomannine and paclitaxel was 3.34, 7.15, 22.60 and 29.12 min, respectively.

Table I. HPLC gradient elution program for paclitaxel quantitation.

Time [min]	A (%)	B (%)
0	95	5
1	95	5
36	82.8	17.2
38	100	0

Every sample was repeated for 3 times and the relative standard deviation (RSD) for all samples was 1–2%. Linearity of the detector response was established for all 4 standards ranging from 0.5 to 250 μg/ml. For HPLC analysis, all solvents and samples were filtered through 0.45 μm filters.

Results and Discussion

For the screening of cultivars with different growth characteristics for their taxane content, needle samples from 17 different cultivars of *Taxus* x *media* were collected from the Secrest Arboretum at The Ohio State University, in which many *Taxus* species and cultivars are collected and planted for research purposes. All needle samples were mixed from multiple trees of each cultivar. The classification of these 17 cultivars is based on the comparison of their growth rate and growing form (Table II). Since our samples were collected from trees growing in similar environment, the influence of environmental factors on taxane production was minimized.

A well-developed taxane HPLC quantitation system (Theodoridis *et al.*, 1998) was employed to analyze the taxane content of these samples. Prior to the HPLC quantitation, the methanol extracts of needles were pre-purified by a solid phase extraction (SPE) cartridge, which removed part of the interfering compounds and facilitated later HPLC analysis.

Large variation was found in the content of 10-DAB, baccatin III, cephalomannine and paclitaxel among these 17 *Taxus* x *media* cultivars (Table II and Fig. 1). For paclitaxel, the content ranged from 108 μg/g extracted dry weight ('Natorp', group 4) to 378 μg/g ('Coleana', group 1). The top three cultivars with the highest paclitaxel content were all found in group 1, the medium-to-fast/upright growth group. Besides 'Coleana', another cultivar in group 1, 'Hicksii', contained the second highest paclitaxel content (322 μg/g), while 'Stovekenii' had the third highest paclitaxel content (309 μg/g).

Another taxane, 10-DAB, is of particular interest in this analysis, since it is the precursor of paclitaxel semi-synthesis. The range of its content was from 203 μg/g ('Hillii' in group 2 and 'Hicksii' in group 1) to 543 μg/g ('Viridis', group 2). The three cultivars with the highest yield of 10-DAB were all found in upright-growing cultivars, with 'Stovekenii' having the second highest 10-DAB content (482 μg/g) and 'Coleana' the third (463 μg/g).

Compared to paclitaxel and 10-DAB, the variation of baccatin III and cephalomannine content was larger. The content range for baccatin III was from 49 μg/g ('Wardii', group 4) to 360 μg/g ('Coleana'). For cephalomannine, it was from 0 (not detected in 'Roseco' of group 3) to 421 μg/g ('Wellesleyana').

On average, group 1 was found to contain the highest content of all 4 taxanes assayed in this re-

Table II. Taxane content of 17 *Taxus* x *media* cultivars in four different growth groups.

Group no. and description	Cultivar name	Taxane content [μg/g]			
		10-DAB	Baccatin III	Cephalomannine	Paclitaxel
1 Medium to fast/upright	Andorea	291	146	125	261
	Coleana	463	360	390	378
	Hicksii	203	88	346	322
	Stovekenii	482	293	60	309
	Wellesleyana	369	72	421	152
2 Slow/upright	Grandfolia	291	55	106	163
	Green Candle	270	248	179	197
	Hillii	203	135	194	234
	Viridis	543	139	169	189
3 Fast/spreading	Fairview	275	102	181	143
	Roseco	238	122	N/D[a]	208
	Runyan	460	51	276	240
	Sebian	422	98	226	148
4 Slow/spreading	Everlow	230	79	188	123
	Kobel	330	134	190	191
	Natorp	378	269	170	108
	Wardii	285	49	75	235

[a] Not detected.

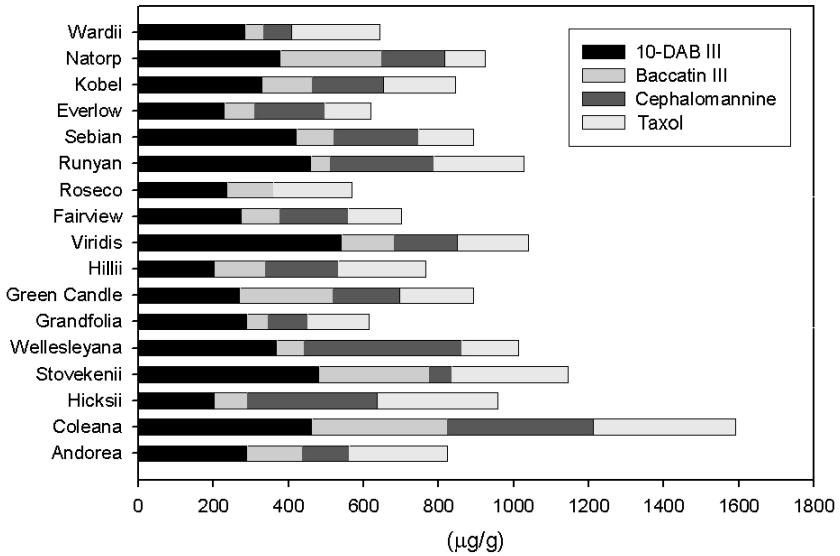

Fig. 1. Content of 10-DAB III, baccatin III, cephalomannine and taxol in *Taxus* x *media* cultivars with different growth characteristics.

port (Fig. 2). Since the cultivars in this group offer advantages over those in other groups, *i.e.*, faster biomass accumulation and applicability of mechanical clippings collection, several cultivars in this group make good candidates for taxane extraction purpose. 'Coleana' contained the highest content of paclitaxel, baccatin III and cephalomannine, as well as the third highest content of 10-DAB. 'Stovekenii' contained the third highest content of paclitaxel and the second highest content of 10-DAB. The wide availability of 'Hicksii', along with its high paclitaxel content, makes it another attractive cultivar.

In this report, we utilized fresh needles as starting material for taxane extraction instead of dried needles. Therefore, our taxane content data was expressed on the basis of extracted dry weight (EDW). This EDW-based taxane content expression was first used by Fett-Neto and DiCosmo (1992). Since degradation of paclitaxel during the drying course of needles was indicated in their report, the EDW-based taxane content offers more accuracy than the commonly used dry-weight-based content. The paclitaxel content obtained in this report is comparable to that of Fett-Neto and DiCosmo (1992). The EDW-based content of paclitaxel in fresh needles of most cultivars in this analysis was higher than that in its original source, *i.e.* the bark of *T. brevifolia* (100 μg/g) on the dry weight basis. 'Hicksii' was found in this report to contain a high amount of paclitaxel, which is in agreement with other authors who based their results on dry weight basis (van Rozendaal *et al.*, 2000; Hansen *et al.*, 1999).

Cultivars of *Taxus* x *media* are very common garden and fence plants. Every year large amount of clippings was pruned from these plants by nurseries. Since needles contain taxanes comparable to their original source (Witherup *et al.*, 1990), these clippings could be utilized to extract paclitaxel and 10-DAB to save patients afflicted by ovarian and breast cancers. By estimation, paclitaxel extracted from 'Hicksii' clippings alone could treat 3,000 to 4,000 ovarian cancer patients annually (Hansen *et al.*, 1999). The cultivars identified by this report could be widely planted for gardening and taxane extraction simultaneously.

Although we found that group 1 on average contained more taxanes than the other groups in this report, there was no report of definite relationship between growth characteristics and taxane accumulation and degradation. Since taxanes are secondary metabolites, this kind of relationship is expected to be indirect if it exists. The influence of various environmental factors on taxane production further complicates this situation. Our results also showed that not all cultivars in group 1 contained a high content of taxanes: 'Wellesleyana' contained a very low content of paclitaxel; 'Hicksii' was also found to contain the lowest 10-

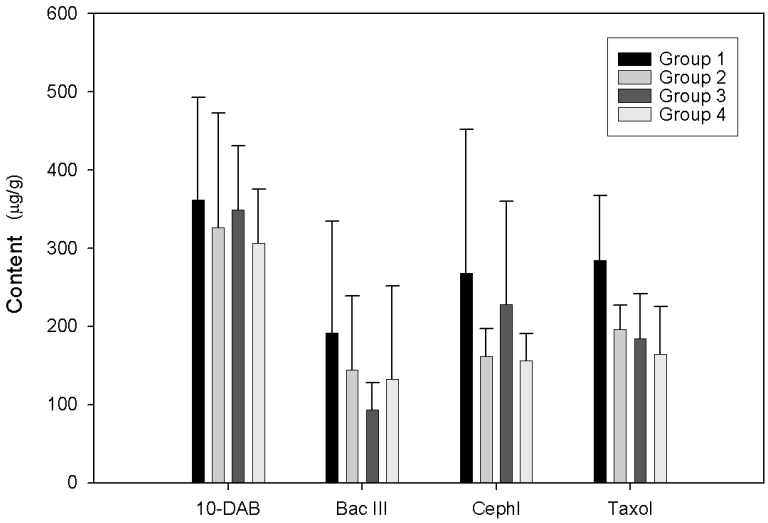

Fig. 2. Average taxoid content in the four growth groups. Group 1, medium to fast/upright; group 2, slow/upright; group 3, fast/spreading; group 4, slow/spreading.

DAB content among the 17 cultivars. By minimizing the influence of environmental factors, the purpose of this report was to identify those cultivars which combine advantageous growing characteristics and high taxane content. In addition to taxane extraction, these cultivars are also good starting materials for developing cell suspension cultures and genetic transformation, which show great potential to develop biotechnology-based systems for paclitaxel production.

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