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Seasonal variation of neutral and basic taxoid contents in shoots of European Yew (*Taxus baccata*)

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Abstract

Seasonal variations of taxoid constituents were determined in shoots of European Yew collected from two locations. The first samples originated from a male *Taxus baccata* tree growing in Gif, France. The second samples were obtained from genetically identical female Irish Yew trees (*T. baccata* var. *fastigiata*), of the same age and growing at one site in Dublin, Ireland. Shoots were collected monthly for one year and separated into needles and stems. Neutral taxoids (paclitaxel and 10-deacetylbaccatin III (10-DAB III)) and basic taxoids (including taxines B) were extracted and quantified. Needles yielded significantly higher levels of taxoids than stems. 10-DAB III contents in needles of French samples showed considerable monthly fluctuations, while in needles of the Irish samples maximum yields of 10-DAB III were found in June. Highest levels of paclitaxel were present between February and April. Basic taxoids occurred in highest concentrations (total alkaloids 9.49 g/kg) in the August collection of French samples, but in needles of the Irish Yew in November and December (total alkaloids 16.9 g/kg; taxines B 10.9 g/kg). No conclusion could be drawn as to the optimum time of year for harvesting, since this varies from tree to tree, depending on *T. baccata* variety, location and taxoid type.

Keywords: *Taxus baccata*; Taxaceae; European Yew; Paclitaxel; 10-Deacetylbaccatin III; Basic taxoids; Taxines B; Anti-tubulin activity; Seasonal variation

1. Introduction

The clinical success of paclitaxel and docetaxel as anticancer agents and the use of 10-DAB III as a precursor in their semi-syntheses, underlines the commercial importance of these neutral taxoids (Guénard, Guéritte-Voegelein & Potier, 1993). Basic taxoids, such as taxine B (the main alkaloid of the needles of the European Yew (Ettouati, Ahond, Poupat & Potier, 1991) and its isomer, are also of future potential value in the semi-synthesis of active derivatives (Poujol, Al Mourabit, Ahond, Poupat & Potier, 1997). Since all these taxoids occur in shoots of the *Taxus* species, it is important to maximize their extraction, for example by knowing the optimum time of year to harvest. A previous paper (Griffin & Hook, 1996) indicated seasonal variations in the paclitaxel content of shoots collected from European Yew. The objective of the present study[1] was to examine this in greater detail, by determining the monthly variations in neutral and basic taxoid contents of shoots taken from two separate collections of *T. baccata*.

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[1] European project AIR3-CT94-1979 entitled "Selection, cultivation and harvesting of yew: silviculture and biotechnological production as alternatives in the development of taxanes with anticancer activity".

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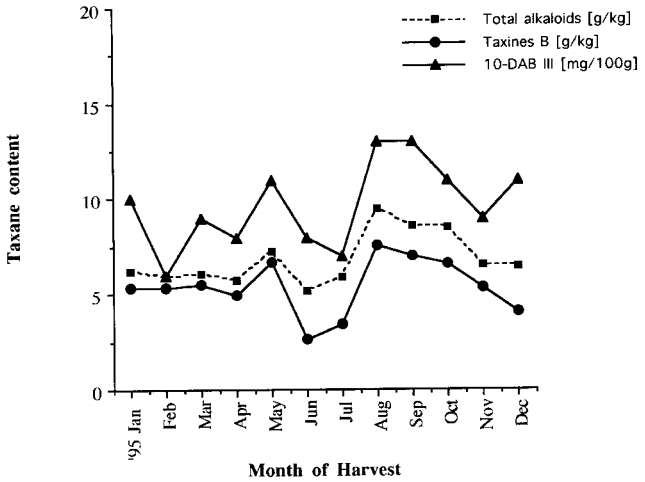

Fig. 1. Seasonal variations of taxoid concentrations in needles of *Taxus baccata* growing in France.

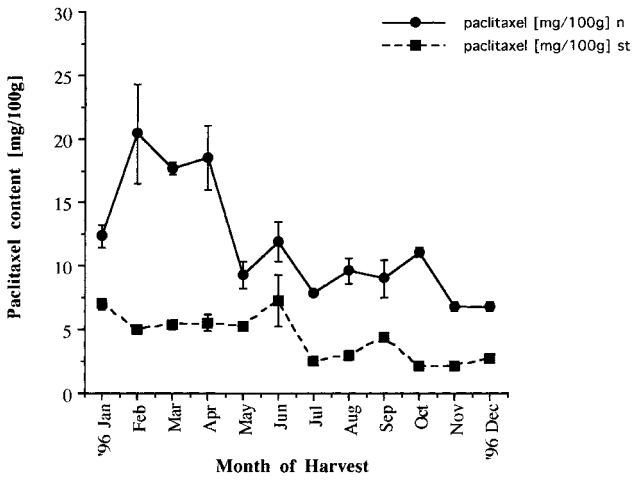

Fig. 2. Seasonal variations in concentrations of Paclitaxel in needles (n) and stems (st) of *Taxus baccata* var. *fastigiata* growing in Ireland.

2. Results and discussion

Plant secondary metabolites, including taxanes, are known to be influenced qualitatively and quantitatively by plant age, variety, season, time of day and weather. The first yew samples in the present study were collected from a male *T. baccata* tree growing near Paris, France. Results are shown in Fig. 1. They indicate considerable seasonal fluctuations with regard to the neutral taxoid 10-DAB III, although August and September gave the highest yields of the samples collected during 1995. Minimum yields of basic taxoids were recorded in June (total alkaloids 5.18 g/kg; taxines B 2.65 g/kg i.e. 51.1% of total alkaloids) and maximum contents in August (total alkaloids 9.49 g/kg; taxines B 7.53 g/kg i.e. 79.4% of total alkaloids). The highest ratios of taxines B to total alkaloids were found between January and May.

For the second collection, shoot samples were taken from genetically identical, female Irish Yew trees (*T. baccata* var. *fastigiata*) of the same age and growing at the same location in Dublin, Ireland. Dried shoots were separated into needles and stems prior to extraction and analyses. Results shown in Figs. 2–4, indicate that needles yield higher amounts of taxoids than stems. This finding, though confirming our previous results for Irish Yew (Griffin & Hook, 1996), is more significant in this study and agrees with results reported for other *Taxus* species (Das & Das, 1994; El Sohly, Croom, Kopycki, Joshi & McChesney, 1997).

Seasonal variations in taxoids were again found to occur. Highest levels of paclitaxel were found present in needles between February and April (Fig. 2). A significant fall in paclitaxel content then occurred between April (190 mg/kg) and May (90 mg/kg), again confirming our previous results for *T. baccata* var. *fas-*

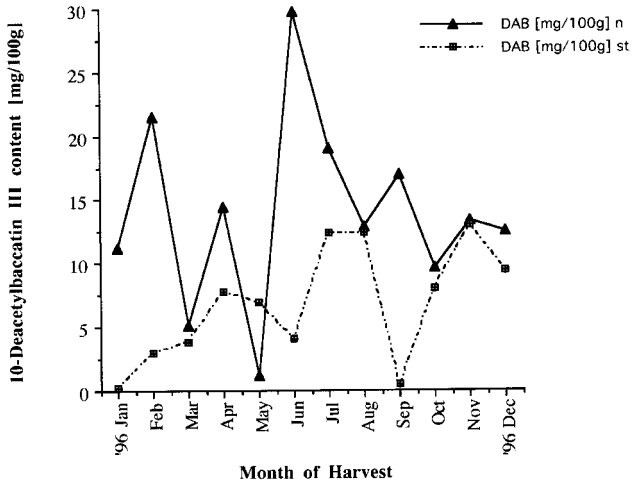

Fig. 3. Seasonal variations in concentrations of 10-deacetylbaccatin III in needles (n) and stems (st) of *Taxus baccata* var. *fastigiata* growing in Ireland.

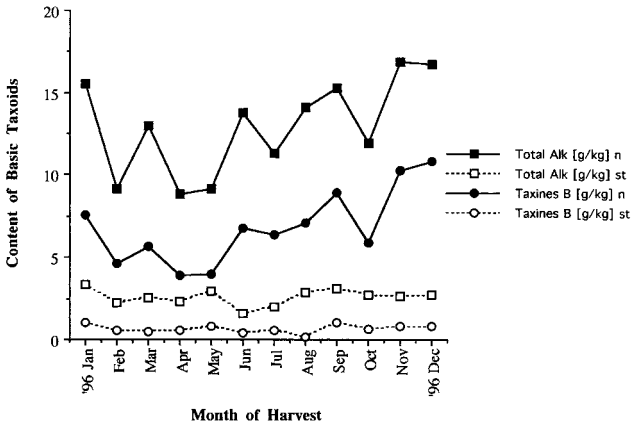

Fig. 4. Seasonal variations in concentrations of total basic taxoids and taxines B in needles (n) and stems (st) of *Taxus baccata* var. *fastigiata* growing in Ireland.

tigiata (Griffin & Hook, 1996). Contents of 10-DAB III in needles were often much greater than those of paclitaxel, with highest concentrations being found in June (298 mg/kg). This agrees with results reported for *T. brevifolia* (Vance, Kelsey & Sabin, 1994) and for *T. x media* 'Hicksii' (El Sohly et al., 1997) who proposed the optimum time for harvesting to be one month before the start of new growth. Basic taxoids (Fig. 4) were found to occur in greatest amounts in needles during winter months, especially November and December (total alkaloids 16.89–16.70 g/kg; taxines B 10.31–10.87 g/kg i.e. 61.1–65.1% of total alkaloids). These results agree with literature references to taxine contents in needles of up to 20 g/kg in winter (Gessner & Orzechowski, 1974) and confirms the fact that yew needles are most toxic to animals during the winter months (Cooper & Johnson, 1984). The highest ratios of taxines B to total alkaloids in needles were found in November and December.

Variations in taxoid levels may be related to flower development on both male and female trees during February and March, to new shoot growth which in Ireland occurs between May and August and to seed maturation which occurs between August and December. Also since paclitaxel production is considered to be plastid-associated (Srinivasan, Ciddi, Bringi & Shuler, 1996), biosynthetic changes in *Taxus* plastids could affect taxoid production. Seasonal variations in carotenoid pigments and xanthophyll fatty acid esters, as well as changes in the ultrastructure of *Taxus baccata* leaf plastids are known to occur (Kufner, Czygan & Schneider, 1978).

Results from the tubulin assays (Fig. 5) carried out on the monthly collections of needles and stems of *T. baccata* var. *fastigiata* showed considerable fluctuations, with maximum activities being recorded in extracts from stems collected in June and August. Stems, though containing lower amounts of paclitaxel, have in general significantly greater 'paclitaxel-like' activity than needles (39% ± 7 vs. 23% ± 6). This could be due to the presence, in stems only, of other active paclitaxel analogues.

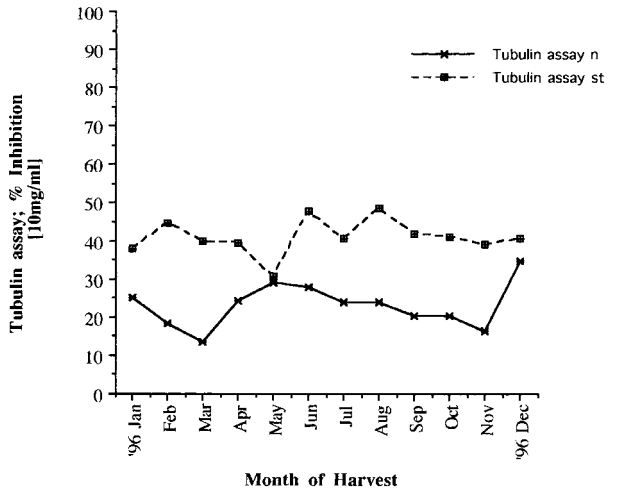

Fig. 5. Seasonal variations in tubulin disassembly (paclitaxel-like) activity in extracts from needles (n) and stems (st) of *Taxus baccata* var. *fastigiata* growing in Ireland.

3. Conclusion

Yields of all taxoids show considerable variation and would appear to be dependent on many factors, e.g. *T. baccata* variety or cultivar, location, weather during the year, soil type and nutrition, etc. For the Irish Yew, results indicate that for maximum paclitaxel yields, shoots should be clipped towards the end of April before the appearance of new growth in May, while a June harvest would give greatest yields of 10-DAB III. Harvesting during the winter months, November to January, would result in greatest amounts of the basic taxoids. In France, highest yields of basic taxoids could result from clippings taken between August and October. From the results it is apparent that there is no single month of the year which is universally ideal for harvesting yew shoots containing maximum amounts of all taxoids.

4. Experimental

4.1. Plant material

1. In France, shoots were collected on the last day of each month during 1995. Samples were taken ca. 1 m above ground level from all sides of a male *T. baccata* L. tree growing in Gif Park, near Paris. Samples were air-dried, needles separated and powdered.
2. In Ireland, genetically identical female trees (×9) of *T. baccata* var. *fastigiata* growing in the grounds of Mount Anville Convent, Dublin were harvested at noon on the last Friday of every month during 1996. Shoots (ca. 20 cm) were clipped from each tree at four sides and at two height levels above ground (ca. 1 and 3 m). Clippings were bulked, dried at < 35°C in a fan-assisted oven and separated into needles and stems before powdering.

4.2. Extraction of neutral taxoids

4.2.1. Paclitaxel

Samples (4 × 3 g) in fine powder form were refluxed with MeOH for 2.5 h, filtered and evaporated to dry-

ness in vacuo. The resultant residue was partitioned between H_2O (10 ml) and EtOAc (10 ml); the EtOAc portion was separated and evaporated to dryness in vacuo.

4.2.2. 10-Deacetylbaccatin III

Extraction protocols were based on (Appendino, Gariboldi, Pisetta, Bombardelli & Gabetta, 1992). Samples (10 g) were extracted six times with 95% EtOH at room temperature, each for 1 h. The resultant extracts were bulked and evaporated to dryness in vacua. This 'ethanolic' extract was partitioned between CH_2Cl_2 and H_2O, with the aqueous fraction then being re-extracted with CH_2Cl_2. The combined organic fractions were dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo at 35°C.

4.3. Extraction of basic taxoids

Extractions were carried out as in Ref. (Ettouati et al., 1991). Samples (50 g) were mixed with NH_4OH and macerated with CH_2Cl_2. The organic extracts were bulked and concentrated under reduced pressure. This concentrated CH_2Cl_2 extract was partitioned to exhaustion with 2% aqu. HCl. The combined acid extracts were washed with heptane, basified to pH 9 with 25% NH_4OH and re-extracted to exhaustion with CH_2Cl_2. The combined organic extracts were washed with H_2O until washings were neutral, then dried over anhydrous Na_2SO_4, filtered and evaporated to dryness in vacuo, to give a 'crude alkaloid' residue.

4.4. HPLC analyses

All solvents used for these analyses were HPLC grade.

4.4.1. Paclitaxel

The residue obtained above was redissolved in MeOH (5 ml) and assayed using a Waters 600 HPLC System. Two columns, with appropriate pre-columns were used during the duration of these experiments: (1) Phenomenex Curosil-G® (4 mm, 4.6 mm × 250 mm) and (2) Waters Symmetry® C_8 (3.5 mm, 4.6 mm × 150 mm).

The mobile phases used were for (1) 10 mM CH_3COONH_4 (adjusted to pH 4 with glacial HOAc)–MeCN–MeOH (56 : 42 : 2), and for (2) 20 mM CH_3COONH_4 (adjusted to pH 5 with glacial HOAc)–MeCN–MeOH–THF (55 : 39 : 4 : 2). The flow rate was 1 ml/min with detection by monitoring absorbance at 227 nm. All analyses were replicated. Quantitations were carried out by reference to a calibration curve prepared on a two times daily basis with dilutions of a pure reference sample of paclitaxel (99.5% purity, Sigma), previously identified by NMR and FTIR (Griffin & Hook, 1996).

The presence of paclitaxel in the extracts was confirmed by TLC and HPLC by co-chromatography with the reference (paclitaxel t_R with Curosil-G 22.7 ± 0.2 min; with Symmetry C_8 21.0 ± 0.4 min).

4.4.2. 10-Deacetylbaccatin III

The residue was redissolved in DMF (20 mg/ml at 10°C) and assayed using a Nova-Pak® C_{18} column (4 mm, 3.9 mm × 150 mm). The mobile phases used were MeOH–H_2O–AcOH (39 : 61 : 0.1) (solvent A) and AcOH (0.1%) in MeOH (solvent B). The elution commenced with solvent A (100%) isocratic for 14 min, then a linear gradient over 10 min to solvent B (100%), which was held isocratic for a further 10 min. The flow rate was 1 ml/min and the t_R for 10-DAB III was 10.7 min. All data were collected over a 200–350 nm range of the absorption spectrum and all chromatograms were plotted at 240 nm (Adeline, Wang, Poupat, Ahond & Potier, 1997).

4.5. HPLC of basic taxoids

The crude alkaloid residue (TA = total alkaloids) was analysed directly by HPLC for taxine B and isotaxine B (taxines B). The residue was redissolved in $CHCl_3$ (5 mg/ml) and injected onto a Nova-Pak® Silica column (4 mm, 3.9 mm × 150 mm). The mobile phase was $CHCl_3$–MeOH–Et_3N [99.4 : 0.5 : 0.1], at a flow rate of 1 ml/min. The t_R for taxine B was 8.9 min and for isotaxine B 11.2 min. All data were collected over a 200–400 nm range of the absorption spectrum and all chromatograms were plotted at 280 nm. Standards of taxines B were prepared as in reference (Adeline et al., 1997).

4.5.1. Tubulin test for 'Paclitaxel-like' activity

Plant material was extracted as for 10-DAB III. Residues were dissolved in DMSO to give a concentration of 10 mg/ml. One μl of this solution was added to 150 μl of the tubulin preparation at 37°C. The mixture was placed in a temperature controlled UV cell at 4°C. Anti-tubulin activity was determined by monitoring the temperature-dependent in vitro assembly of microtubules by observing the turbidimetric response at 350 nm (Shelanski, Gaskin & Cantor, 1973; Lataste, Senilh, Wright, Guénard & Potier, 1984).

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