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Taxol content in the bark of Himalayan Yew in relation to tree age and sex

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Abstract

Taxol content in the bark of *Taxus baccata* trees growing in a homogenous (uniform) environment at Jageshwar, District Almora in Central Himalaya has been quantified. The average taxol concentration in the bark of sampled trees was $0.0558 \pm 0.008\%$ (of dry wt.) and was about 64% higher for male plants (averaged across tree age) in comparison to female trees. Maximum taxol content was recorded in the bark samples collected from trees of >110 yrs age. ANOVA indicates a significant difference in the taxol content of bark from trees of different ages, however, differences were not significant between sexes. Taxol was quantified by HPLC using a standard curve prepared with authentic taxol; the identification of bark taxol was confirmed by UV and mass spectrometry. The total taxol content of the bark of *Taxus* trees across an age series was found to range between 0.064 to 8.032 g/tree, and a tree of about 100 yrs age can yield 5.74 kg dry bark.

Keywords: *Taxus baccata*; Taxaceae; HPLC; Taxol; Tree age; Bark biomass

1. Introduction

The diterpenoid alkaloid, taxol® (paclitaxel), first isolated from the bark of *Taxus brevifolia* Nutt. (Wani et al., 1971) is the most important discovery in human chemotherapy, and currently used for the treatment of several forms of breast, liver, lung, blood and gynaecological cancers (McGuire et al., 1989; Rowinsky et al., 1990; Holmes et al., 1991; Pezzuto, 1996; Yuan et al., 2000). Subsequently, taxol and taxoid derivatives have been reported from foliage and bark of several other species of *Taxus* like *T. baccata, T. canadensis, T. cuspidata, T. yunnanesis*, etc. (Witherup et al., 1990; Fett-Neto and DiCosmo, 1992; ElSohly et al., 1995; Singh et al., 1997). Although, complete chemical synthesis of taxol is now possible (Holton et al., 1994; Nicolaou et al., 1994), the method is uneconomic (Collin, 2001). At present, most taxol is prepared by semisynthesis from baccatin III or 10-deacetylbaccatin, precursors to taxol, and also isolated from slow growing yew trees (Collin, 2001).

T. baccata L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan Yew) is the only species of *Taxus* found in the Indian temperate Himalaya and in the hills of Meghalaya, Nagaland and Manipur at an altitude of 1800–3300 m amsl. In recent years, its survival has been threatened due to uncontrolled harvesting for the extraction of the drug taxol (Rikhari et al., 1998), but also on account of removal of old forests (Rikhari et al., 2000) in the Indian region. It is a medium sized slow growing evergreen gymnosperm and the plant has been in use since long in the traditional systems of Unani and Ayurvedic medicine (Anon., 1976). The commercial supply of taxol still depends on the natural sources and reports indicate that the levels vary in different plant parts (Witherup et al., 1990). There is, however, general paucity of information from the Indian Himalayan region regarding taxol content in different parts of *T. baccata* trees, except for a few studies on the identification of taxol and other taxanes in *T. baccata* (Das et al., 1995; Singh et al., 1997). It was, therefore, thought relevant to evaluate the effect of tree age and sex on taxol levels in the bark of *T. baccata* trees growing in a nearby area at Jageshwar, and to estimate the total bark biomass and taxol content/plant. Variation in the taxol content of bark from male and female trees of different

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ages growing in a homogenous (i.e., uniform) environment has also been determined. This information can be useful in the selection of elite plants not only from the present study site but also from other provenances for mass scale propagation of high yielding plants for cultivation, and for proper management and conservation of the species.

2. Results and discussion

The study indicates considerable variation in the taxol content of bark within the natural population of *T. baccata* trees growing in the Jageshwar area (Table 1). Based on ANOVA a significant difference can be seen in the taxol content of bark collected from individual trees of different ages. Although, across the age series bark samples from male trees (0.0376–0.1167%) had higher taxol concentration in comparison to samples from female trees (0.0129–0.0810%), the values were not significantly different (Table 1). Significant differences were also not observed for taxol content of dark coloured bark with secondary growth from male and female trees (Fett-Neto and DiCosmo, 1992). The bark from young trees of either sex contained higher concentration (0.0324–0.0655%) of taxol as compared to mature trees; however, maximum (avg. 0.1155%) taxol levels were recorded for older trees (>110 yrs of age). The large differences observed amongst individual trees of *T. baccata* (Table 1) indicate genotypic variation and further suggest that opportunities exist to apply strong selection pressure in identifying superior individuals/trees for taxol content (Wheeler et al., 1992). While nothing is known about the heritability of taxane content, studies by Kelsey and Vance (1992) have also reported genetic variation in the taxol concentration of bark amongst individual trees growing within a fairly uniform environment and the variations as large as 45–60% was observed. Further, samples from different geographical locations are likely to have even greater variations.

The average taxol content of 12 trees (based on bark samples from six male and six female trees) was 0.0558% (coefficient of variation = 60.5%) whereas the average levels for male and female trees were 0.0694 and 0.0424%, respectively. The coefficient of variation was also large for male and female trees (50.9 and 65.4%, respectively). The amount of taxol (on a dry wt. basis) detected in the bark was either comparable to the reported levels from *T. brevifolia, T. baccata, T. canadensis* and *T. cuspidata*×*media* cvs. Capitata, Densiformis and Hicksii (Wani et al., 1971; Witherup et al., 1990) or higher in comparison to that from *T. brevifolia* [0.001–0.01% in April and 0.001–0.006% in September, Wheeler et al. (1992); 0.0146–0.0163%, ElSohly et al. (1995); 0.007%, Collin (2001)] and *T. cuspidata* [0.011–0.031%, Fett-Neto and DiCosmo (1992)]. ElSohly et al. (1995) have demonstrated that needles of ornamental *Taxus* are also rich in taxane and surpass the levels reported from the bark of *T. brevifolia*. It may be mentioned that the taxol content of needles was not estimated in the present study.

The identity of taxol isolated from the bark of *T. baccata* was confirmed by fast atom bombardment (FAB) mass spectrometry. The UV spectrum recorded for the HPLC purified material from bark samples was identical with the standard taxol. Ratio of absorbance at 224 and 196 nm was 0.32 in case of taxol isolated from samples, whereas it was 0.33 for the standard taxol. The mass spectrum of taxol purified from the bark was in agreement with that of authentic taxol. The spectrum showed an abundant protonated molecule at m/z 854, sodium ion at m/z 876 and characteristic fragment ions at m/z 509 and 569 also seen with authentic taxol. On the basis of three spiked samples, the loss during extraction and purification was estimated to be 17, 15, 18% (average 16.7%); the results presented in Table 1 have been appropriately corrected for purification losses.

Tree age was found to be positively related to the total bark biomass and taxol content (Table 2; $P<0.01$).

Table 1
Taxol content (% of dry wt.) in the bark of *T. baccata*[a]

Age[b] class (arbitrary)	Male trees		Female trees	
	Age (yrs)	Taxol (%)	Age (yrs)	Taxol (%)
Young trees	40	0.0376	44	0.0324
	52	0.0516	56	0.0335
	57	0.0655	59	0.0345
Mean		0.0512		0.0333
Mature trees	96	0.0400	84	0.0156
	97	0.0414	99	0.0200
	108	0.0429	109	0.0129
Mean		0.0417		0.0161
Old trees	126	0.1151	125	0.0807
	140	0.1167	133	0.0714
	161	0.1136	153	0.0810
Mean		0.1155		0.0778
LSD at 5%		0.0001		0.0001

Analysis of variance (ANOVA)

Source of variation	d.f.	MS	F-ratio	*P*
Between age	8	0.001376	3.25	0.017
Between sex	1	0.002312	4.63	0.090
Total	17			

[a] The bark samples were taken from live standing trees in the month of September, 1998.

[b] Age was calculated on the basis of bole radius.

This study indicates that a tree of about 100 yrs age can yield 5.74 kg bark of which the bole accounts for about 72%. The average taxol content for trees of different ages was 0.1624 g/tree; however, the taxol content was found in the range of 0.064 g/tree (27 yrs old tree) to 8.038 g/tree (136 yrs old tree) in which contribution of the bole bark was 65 and 75%, respectively. Taxol content for trees of > 110 yrs age was higher than the values (200–500 mg/tree) reported for *T. brevifolia* by Rowinsky et al. (1990). Approximately 3–6 trees of *T. brevifolia* are required to obtain 1.0 g taxol needed for the treatment of one patient (Cragg et al., 1993).

It can be concluded that a significant variation in the bark taxol content exists depending upon the age of trees growing within the same environment. This difference would appear to be related to genetic variation amongst individual trees. Total bark biomass and taxol content was found to increase with increase in tree age. The study could be useful for developing an efficient and effective management approach to fulfil the demand of taxol and for *in situ* conservation as the species is under pressure because of excessive harvesting as well as degradation of its habitat for various reasons (Rikhari et al., 2000). The species has been included in the list of critically rare plant species by IUCN (Samant et al., 1998). Further, the species exhibits poor recovery because of extremely slow growing nature, poor seed germination and canopy damage (Rikhari et al., 1998). Thus, any form of severe disturbance may cause loss of the species. In order to meet the conservation objectives, clones with higher taxol content need to be identified from various provenances for clonal propagation for which a simple, inexpensive and efficient technique has been developed (Nandi et al., 1996, 1997). This should pave the way for mass scale propagation needed to initiate cultivation for harvesting of biomass for taxol, as in the USA (Wheeler and Piesch, 1993). This should also help to conserve the genetic diversity at the ecosystem level by eliminating the pressure from natural vegetation.

Table 2
Bark biomass and taxol content (both on dry wt. basis) along an age series of *T. baccata*[a]

Age (yrs)[b]	Bark biomass (kg/plant)		Taxol (g/plant)		
	Bole	Branch	Bole	Branch	Total
27	0.10	0.05	0.043	0.021	0.064
34	0.42	0.19	0.179	0.081	0.260
36	0.46	0.22	0.195	0.094	0.289
38	0.55	0.26	0.233	0.111	0.344
48	0.79	0.39	0.336	0.166	0.502
51	0.95	0.48	0.404	0.204	0.608
53	1.14	0.55	0.485	0.233	0.718
65	1.48	0.73	0.629	0.311	0.940
68	1.89	0.91	0.539	0.260	0.791
74	2.36	1.08	0.673	0.308	0.981
76	2.63	1.20	0.750	0.342	1.092
89	3.18	1.38	0.907	0.394	1.301
101	4.12	1.62	1.175	0.402	1.577
114	5.11	1.90	5.004	1.861	6.865
136	6.11	2.05	6.025	2.007	8.032

[a] The samples were taken from fallen trees in the month of March, 1995.

[b] Age was calculated on the basis of annual rings (9.5 rings per cm of bole radius).

3. Experimental

3.1. Plant material and bark collection

In the Jageshwar area (29°35′–29°39′ N and 79°59′–79°53′ E), District Almora of Central Himalaya, *Taxus baccata* subsp. *wallichiana* (family Taxaceae, order Taxales) grows between 1790 and 1950 m amsl. The total area under its habitat is approximately 120 ha. Age of individual tree was calculated by multiplying the radius at ground level (RGL) of individual tree with 9.5 rings/cm (unpublished results). Bark samples (250 g fresh weight, from male and female trees) from a hectare of homogenous (i.e., uniform) site were collected during September, 1998. The air dried (in shade) bark samples were finely powdered in a blender. The dry weight (% of fresh weight) of air dried bark samples taken from trees of various girth classes ranged from 39.24 to 47.80%. If the bark samples were dried in an oven at 60 °C upto constant weight, the dry weight values ranged between 39.05 to 45.40%. There was no appreciable difference in the moisture content of bark samples from male and female trees.

3.2. Extraction

Extraction was carried out following the method of Grothaus et al. (1993) with partial modification. In brief, powdered bark samples (10.0 g) were first extracted in 100 ml methanol (twice at 24 h interval) and the extracts were filtered through glass wool. The filtrates were dried in vacuo (30 °C) in a rotatory film evaporator (Polymix, Kinematica, Switzerland), residue taken up in 50 ml of 90% (v/v) methanol and partitioned with hexane (3× equal volume); the methanol fraction was reconstituted with an equal volume of distilled water (50 ml) and partitioned with dichloromethane (3× equal volume). The dichloromethane fractions were combined, dried in vacuo (30 °C) and residue taken up in chloroform for further chromatography. To estimate the extraction losses, some samples were spiked with known quantity of standard taxol (Sigma). The per cent loss was calculated on the basis of recovery of added taxol (following HPLC) by subtracting the values from unspiked samples.

3.3. Column chromatography

Column chromatography was carried out on a glass column (30×2 cm i.d.) packed with silica gel (60–120 mesh; Qualigens Fine Chemical, Mumbai) to 12 cm bed volume. It was regenerated in $CHCl_3$ and washed several times (3×) prior to use. Samples (dissolved in $CHCl_3$) were loaded on column and washed (2×) with $CHCl_3$. Taxanes were eluted in acetonitrile (2-bed volume) and the eluate was dried in vacuo and taken up in HPLC grade methanol (MeOH) for HPLC analysis.

3.4. HPLC analysis

Quantification was carried out by subjecting purified samples (20 μl) to HPLC analysis (Kontron 322, Italy) equipped with a reverse phase column (Spherisorb ODS-2; 250×4.6 mm i.d., 5.0 μm). Taxol was eluted in an isocratic mode with MeOH:AcN:H_2O (20:40:40 v/v) at a flow rate of 1.2 ml/min and the column eluate was monitored at 227 nm with an online UV detector. Taxol was quantified on the basis of standard curve prepared by injecting known quantities of authentic taxol. The UV spectrum (190–360 nm) of HPLC purified taxol from bark samples was recorded with a spectrophotometer (UVIKON 931, Italy) and compared with that of authentic taxol (Sigma Chem. Co., St. Louis, USA). The ratio of the absorbance at 224 and 196 was also compared with authentic taxol.

3.5. Mass spectrometry

Fast atom bombardment (FAB) mass spectra of HPLC purified samples and standard taxol were carried out at the Indian Institute of Chemical Technology, Hyderabad, India on glycerol:dithiothreitol: dithioerythritol (8:5:3 v/v/v) matrix.

3.6. Tree age and calculation of bark biomass

Tree fall and canopy damage due to heavy snowfall is common in the higher altitudes of Central Himalaya. Immediately after snow melt (March end, 1995) freshly uprooted *Taxus* trees (with relatively undamaged canopy) were selected for calculation of tree biomass. The selected trees represented a wide girth range and the age of the individual tree was calculated by counting the annual growth rings (Chaturvedi and Singh, 1982). It may be mentioned that the sex of these fallen trees could not be determined and, therefore, the values given in Table 2 can not be ascribed to male or female trees. Circumference ($C=2\pi r$) and bark thickness at 3–5 points, and length (ℓ) of selected fallen trees were measured to calculate bole volume using the formula $\pi r^2 \ell$. The bark volume was calculated by subtracting total bole volume (with bark) from bole wood volume (without bark). Square pieces of bark were removed from 3–5 places along the bole length to calculate the bark specific gravity. The bark volume was multiplied by bark specific gravity for estimation of total bole bark biomass. Branch bark biomass was calculated by developing wood to bark ratio of branches of different thickness.

3.7. Statistical treatments

The experiment was designed as a split-plot, where the age was the experimental unit of the entire tree and sex was the experimental unit of subplot. The various statistical treatments were carried out following Microsoft Excel programme, and Snedecor and Cochran (1967).

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