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TAXOL CONTENT IN THE SEEDS OF *TAXUS* SPP.

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Key Word Index—*Taxus cuspidata*; *T. baccata*; *T. brevifolia*; Taxaceae; yew; mature seed; immature seed; taxane; taxol; taxol fluctuation.

Abstract—The concentrations of taxol and related compounds in the mature dried seeds of several *Taxus* species were determined by HPLC. In addition, the taxol level of the seeds of *T. cuspidata* at different stages of maturation was studied. The variation in the taxane content was dependent on individual trees within species as well as among species. The average contents of taxol and its precursor, 10-deacetyl baccatin III (10-DAB III) in the mature seeds of different species were 67.3 ± 50 and 96.2 ± 83 $\mu g\,g^{-1}$ dry wt, respectively. In mature seed parts of *T. cuspidata* var. *latifolia* obtained from tree U5, the embryo weight was *ca* 0.2% of that of the dry weight of the whole seed and contained the highest level of taxol (894 $\mu g\,g^{-1}$) when compared with that obtained for the endosperm (23 $\mu g\,g^{-1}$) and testa (376 $\mu g\,g^{-1}$). However, the taxol content per seed was highest in the testa (20.5 μg) followed by the endosperm (0.75 μg) and embryo (0.18 μg). The taxol content of the fresh seed reached a maximum of 332 $\mu g\,g^{-1}$ and 20 μg seed^{-1} at the middle stage of seed maturation and then decreased with further maturation. The taxol identification in the seed was confirmed by electrospray mass spectrometry.

INTRODUCTION

Taxol, a diterpene amide, originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) [1] is considered currently the most promising chemotherapeutic agent for the treatment of ovarian cancer [2]. It has a unique mode of action on the microtubulin protein responsible for the formation of the spindle during cell division. Unlike other spindle poisons such as vinblastine or colchicine, which both prevent the assembly of tubulin, taxol promotes the polymerization process but inhibits the depolymerization [3]. However, the limited supply of this compound hinders the treatment of patients with ovarian and other forms of cancer. Pacific yew has a slow growth rate and the removal of the bark results in death of the tree. The increasing demand for this compound may cause devastation of the Pacific Northwest forest [4]. Recently, it has been reported that taxol was also produced by *Taxomyces andreanae*, an endophytic fungus of the Pacific yew tree [5]. Meanwhile, the biosynthesis of taxol has not been as well studied compared to its development as an anticancer agent [6, 7].

On the other hand, gibberellins (GAs) are well known diterpenoid plant growth hormones, which were originally isolated from fungi in 1938 [8], and later found in plants [9]. The immature seeds of various plants are known to be rich sources of GAs [10]. Thus, the immature seeds have been used to study the GA biosynthesis in cell-free systems [11]. In this context, it could be assumed from the research of GA biosynthesis using immature seeds that yew seed will be an ideal plant organ to study the biosynthesis of taxol. Since little is known concerning the endogenous level of taxol and related compounds in the developing yew seeds [12], this work was undertaken to determine the concentrations of taxol and related compounds in the seeds of *Taxus* species, as well as to study the fluctuations of taxol during the seed maturation.

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RESULTS AND DISCUSSION

The levels of taxol and three related compounds in the whole mature dried seeds of various *Taxus* species are shown in Fig. 1. The taxane content varied in individual trees within species as well as among species, showing the genetic diversity in yew spp. The average content of taxol and its precursor, 10-deacetyl baccatin III (10-DAB III) in mature seeds collected from different *Taxus* species was 67.3 ± 50 and 96.2 ± 83 $\mu g\,g^{-1}$ dry wt, respectively, whereas the mean seed wt was 59.7 ± 9.7 mg. The U5 yew seeds, which were collected from *T. cuspidata* var. *latifolia* grown in Ullung Island, contained the highest level of taxanes including taxol and 10-DAB III, 0.018 and 0.035% dry wt. respectively.

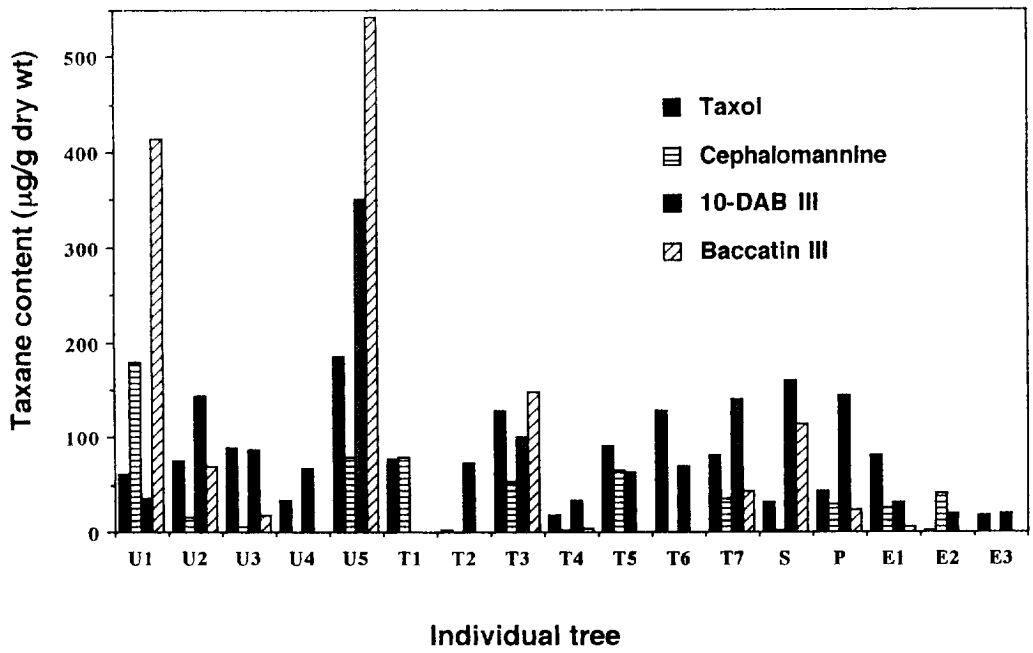

Fig. 1. The concentrations of taxol and related compounds in the mature dried seeds of various *Taxus* species, (U) Seeds of *T. cuspidata* var. *latifolia* collected from Ullung Island, (S) seeds of *T. cuspidata* collected from Mt. Sobaek, (E) seeds of English yew (*T. baccata*), (P) seeds of Pacific yew (*T. brevifolia*), (T) seeds of ornamental yews (*T. cuspidata*) collected at Taejon area.

Table 1. Average taxol concentrations in the different parts of 50 mature seeds collected from yew tree U5, *T. cuspidata* var. *latifolia* and yew tree T3, *T. cuspidata*, respectively

Yew tree	seed part	Dry wt seed^{-1} (mg)	Taxol g^{-1} dry wt (μg)	Taxol seed^{-1} (μg)
Tree U5	Embryo	0.2	893.7	0.18
	Endosperm	33.4	22.5	0.75
	Testa	54.4	376.2	20.47
	Whole seed	88.0	243.2	21.40
Tree T3	Embryo	0.2	184.0	0.04
	Endosperm	25.1	70.5	1.77
	Testa	43.2	162.4	7.02
	Whole seed	68.5	128.8	8.83

The taxol concentrations in the embryo, endosperm and testa separated from the mature dried seeds of tree U5 were analysed and their levels are shown in Table 1. The data indicated that the embryo tissue, with a weight of *ca* 0.2% of that of the whole seed, had the highest taxol level (893 μg) when compared with that of the endosperm (23 μg) and testa (376 μg) based on gram dry wt. However, the taxol content per seed was much higher in the testa (20.5 μg or *ca* 95.6% of the total taxol content in the seed), whereas the embryo and endosperm contained only 0.18 and 0.75 μg, respectively. Similar results were obtained for the mature dried seeds of *T. cuspidata* collected from tree T3 and are shown in Table 1.

The identity of the isolated taxol from the seeds was confirmed by electrospray mass spectrometry. The mass spectrum of taxol purified from the seed was essentially the same as that of the authentic sample. The mass spectrum of taxol showed an abundant protonated molecule at *m/z* 854 and a sodium addition ion at *m/z* 876 suitable for identification.

The taxol content of the seeds of ornamental *T. cuspidata* (tree T3), collected at different stages of maturation, is presented in Fig. 2. The fluctuations of taxol level calculated per fresh seed and per gram fresh weight showed almost the same patterns during the seed maturation. The very immature seeds collected on 6 August contained a relatively high level of taxol, with 9 μg fr. seed^{-1} and 125 μg g^{-1} fr. wt. The taxol content dropped and then subsequently increased from 26 August and reached a maximum level by 28 September correspond-

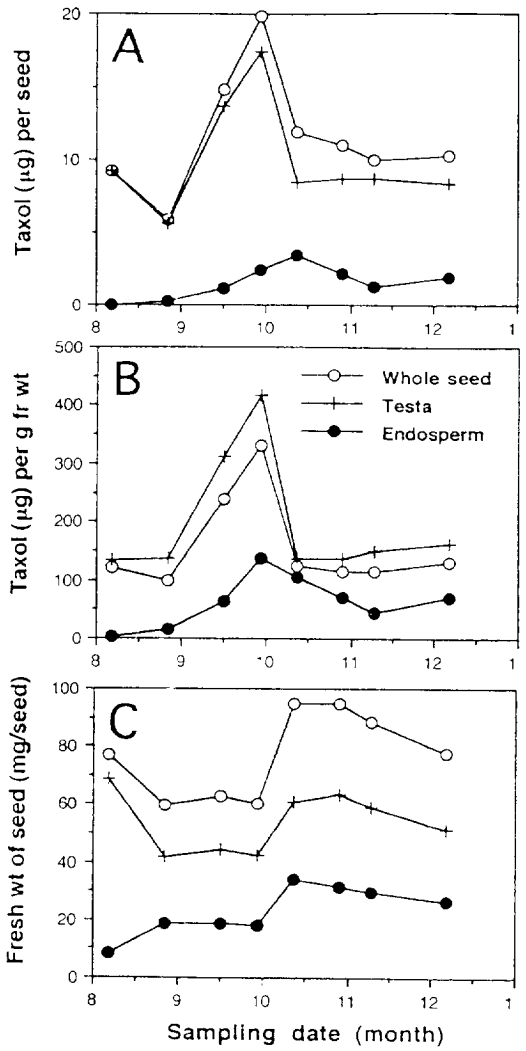

Fig. 2. Taxol level in the seeds of ornamental *T. cuspidata* at different stages of growth. (A) Taxol content per seed; (B) taxol content per g fresh wt; (C) growth curves of seeds of *T. cuspidata* (tree T3). The taxol curve of the embryo was not determined owing to difficulty in separating the smaller liquid embryo from the immature seeds.

ing with the middle stage of the seed development, and then decreased with further maturation. The maximum taxol level was 332 μg and 416 μg g^{-1} fr. wt for the whole seed and the testa, and 19.9 μg and 17.4 μg fr. $seed^{-1}$ for the whole seed and the testa. The green colour of the aril surrounding the seed turned to slight red from early September and to complete red by late December.

Similar phenomena were observed in the levels of GAs of the developing seeds of *Phaseolus vulgaris* [13] and *Pisum sativum* [14]. The endogenous level of GA_1 in the seeds of *P. vulgaris*, which is the physiologically active major GA, showed maximum level (8 μg $seed^{-1}$) in the middle stage of maturation and decreased during seed maturation. The activity of GA 3β-hydroxylase converting GA_{20} to GA_1 reached a maximum at 21 days after flowering, corresponding with the middle stage of maturation, and subsequently decreased during seed maturation of *P. vulgaris* [15]. Furthermore, GA 3β-hydroxylase was purified and characterized from immature seeds of *P. vulgaris* [16].

After comparing the taxol fluctuations with that of GAs during seed development, we suggest that the immature seeds of *Taxus* species can be a good source for the preparation of a cell-free system to study the biosynthetic pathway of taxol.

EXPERIMENTAL

General. HPLC: Curosil-B (Phenomenex, Torrance, CA) column (250 × 3.2 mm, 5 μm), mobile phase 10 mM NH_4OAc (pH 4.0)–MeCN (11:9), flow rate 0.6 ml min^{-1}. The eluate was monitored at 230 nm using a Spectra 100 variable wavelength, UV-visible detector (Spectra-Physics Inc., San Jose, CA). MS: electrospray mass spectrometer (VG Quattro Triple Quadrupole Mass Spectrometer, Fisons Instruments, U.K.), mobile phase MeOH–H_2O (2:3) containing 0.05% HCO_2H, flow rate 10 μl min^{-1}. The sample (*ca* 0.5 μg) was loop injected under a capillary voltage, 3.0 kV and source temp. 70°.

Plant materials. The seeds of *T. cuspidata* and *T. cuspidata* var. *latifolia* were collected in Korea in 1993 from natural yew habitats of Mt. Sobaek and Ullung Island, respectively. For the analysis of taxol in the different parts of seeds: the embryo, endosperm and testa were separated from fifty mature dried seeds collected from *T. cuspidata* var. *latifolia* (U5 Tree) and *T. cuspidata* (Tree T3). Cultivated *T. cuspidata* tree (T3) was used for harvesting the seeds, at different stages of growth, between August and December 1993. The mature seeds of Pacific yew (*T. brevifolia*) were collected from the Rogue River National Forest in Jackson County, southwestern Oregon in September 1992 by Dr P. W. Owston (Forestry Sciences Lab., Pacific Northwest Research Station, USDA), whereas those of English yew (*T. baccata*) were collected from the Seattle area, Washington by Dr K. H. Han (University of Washington). The taxonomic identification was verified by Prof. Byung-Yun Sun (Director of Herbarium, Chonbuk National University, Chonju, Korea) and voucher specimens are deposited in the Herbarium. The immature seeds were directly analysed after collection, whereas the mature dried seeds were kept at 0° after collection until analysis.

Analysis of taxol and related compounds. Endogenous taxanes were extracted, purified and quantitatively analysed by a modified method of ref [17]. Mature dried seeds (1 g) of various *Taxus* species were extracted twice with 20 ml CH_2Cl_2–MeOH (1:1) for 12 hr after hexane treatment to remove nonpolar components. For analysis of the taxol level of the seeds at different stages of maturation, immature fresh seeds (1 g) were extracted in the same manner. The combined extracts were evaporated to dryness, and then partitioned twice with CH_2Cl_2 and H_2O (20 ml each). The CH_2Cl_2 soluble fraction was dried *in vacuo*, and filtered through a 0.5 μm FH-type Mil-

lipore filter after redissolving in 1 ml MeOH. The filtrate (10 μl) was loaded onto a Curosil-B column (250 × 3.2 mm, 5 μm) connected with a Spectra-Physics HPLC system, and eluted with a mixture of 10 mM NH_4OAc (pH 4.0) and MeCN (11:9) at a flow rate of 0.6 ml min^{-1}. The taxanes were detected at 230 nm. The retention times (min) of taxol, cephalomannine, baccatin III and 10-DAB III were 13.0, 10.8, 5.2 and 3.8, respectively. Spiking of the CH_2Cl_2 soluble fraction of the seed with standards taxol, cephalomannine, baccatin III and 10-DAB III resulted in a signal enhancement at the R_t values of these taxanes. The quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic taxanes. The data are expressed as an average of two replicates. The correlation coefficient (r) for the four taxanes studied (concentration range 100–1500 μg) was over 0.98. One sample was analysed three times independently to measure the deviation among analyses. The coefficient of variation (%) for these three replicates was $\pm$ 0.001.

The qualitative analysis of taxol was done by electrospray mass spectrometry. The taxol fraction separated by HPLC was collected several times and concentrated. This purified taxol fraction (*ca* 0.5 μg) was loop injected and eluted with solvent (MeOH–H_2O, 2:3) containing 0.05% formic acid at a flow rate of 10 μl min^{-1} under a capillary voltage, 3.0 kV and source temperature, 70° [18, 19].

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