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TAXOL AND CEPHALOMANNINE CONCENTRATIONS IN THE FOLIAGE AND BARK OF SHADE-GROWN AND SUN-EXPOSED *TAXUS BREVIFOLIA* TREES

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ABSTRACT.—Taxol and cephalomannine concentrations were measured in the bark and foliage of Pacific yew trees growing in the shade of a forest canopy and at a site nearby where trees had been exposed to full sunlight for 6 years. Bark was the only tissue showing concentration differences due to light, with significantly greater quantities of both compounds in the bark of shaded trees than in the bark of sun-exposed trees. In either light regimen taxol concentrations were greater in the bark than in the needles or twigs. Taxol concentrations were higher in older needles than in younger needles, and the reverse was observed for twigs. Cephalomannine was substantially lower than taxol in concentration. Cephalomannine varied similarly to taxol among tissues of shade trees but not among tissues of sun-exposed trees.

The rapid development of taxol for use as an anticancer drug has been hindered substantially by its limited supply (1). Currently, the only source for this drug approved by the Food and Drug Administration is from the bark of Pacific yew, *Taxus brevifolia* Nutt. (Taxaceae) (2), a slow-growing tree native to the mixed coniferous-hardwood forests of the Pacific Northwest. In the Cascade and Coast ranges of Washington and Oregon this tree is typically found in small groups or as individuals scattered infrequently under the canopy of the dominant species where it has adapted to the low light of the understory (3). It is more abundant in some areas of southern Oregon and the northern Rocky Mountains as in north central Idaho where it occurs as the dominant species in some forests (4). Factors limiting the taxol supply are Pacific yew's low abundance, slow growth, small stature, and thin bark, and low taxol concentrations in all the tissues. The production of 1 kg of taxol requires bark from approximately 2000–3000 trees (5).

The natural supply of Pacific yew is limited, and if this species is to continue as a major source of taxol into the future, trees will probably have to be cultivated. Very little is known about the genetic and environmental influences on the production of taxol and other taxanes. Cultivated trees are grown under less shady conditions than those usually occurring in the forest. Consequently, it is not possible to predict how various cultivation practices will influence the production of these compounds relative to native trees in the forest. In this paper we report how increased light, after removal of the forest canopy, has affected the concentration of taxol and cephalomannine in the foliage and bark of Pacific yew.

In 1984, five hectares of Douglas fir overstory was removed for salvage in the McDonald Experimental Forest at Corvallis, Oregon, exposing the Pacific yew understory trees to full sunlight. In December of 1990 after six years of growth in full light, ten trees were tagged and the stem bark, needles and twigs from the first terminal node, and older needles and twigs from the second node were sampled for analysis of their taxol and cephalomannine concentrations. About 800 m away, under the shade of an undisturbed forest canopy, ten other trees of similar diameter were tagged and sampled for comparison.

RESULTS AND DISCUSSION

Six years of exposure to full light affected canopy and bark structure of trees growing in the stand from which the overstory had been removed. Tree bark, including the

phloem tissue, and the sapwood or water-conducting tissue, was significantly thicker in the sun-exposed than in the shaded trees (Table 1). Specific leaf area (leaf area: dry wt) (6) was significantly greater in the sun-exposed than in the shaded trees. The difference in specific leaf area was due to an increase in mass rather than a decrease in leaf area of the sun-adapted needles (Table 1). In addition, sun-exposed branches had a greater proportion of their first node foliar biomass as needles than did the shaded trees (3.83 vs 3.16, g needles/g twigs, $p \leq 0.0001$).

TABLE 1. Structural Characteristics of *Taxus brevifolia* Trees Grown Under a Forest Canopy (Shade) and With Canopy Removed for Six Years (Sun).[a]

Structural characteristic	Light status	
	Sun	Shade
Stem diameter (cm)	20.3 ± 2.1	17.2 ± 1.8 ns
Sapwood ratio[b]	0.13 ± 0.01	0.08 ± 0.02
Bark thickness (mm)	4.32 ± 0.26	2.70 ± 0.16
Needle dry wt (g·20 needles)	0.122 ± 0.008	0.088 ± 0.007
Needle area (cm^2·20 needles)	17.25 ± 0.95	17.89 ± 1.17 ns
Specific leaf area ($cm^2 \cdot g^{-1}$)	142.6 ± 6.0	206.4 ± 6.8
Photosynthetic photon flux density ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	678.8 ± 97.4	27.1 ± 5.1

[a]Differences between means detected by Scheffe's test, $p \leq 0.05$, n = 20, ns = no significant difference detected. Entries are mean ± standard error of means.

[b]Ratio of sapwood to combined sapwood-heartwood diameter.

Analysis of taxol extracted from the different tissues of the 20 sampled trees indicated that only taxol concentrations in the bark differed between the shaded and unshaded trees. Bark from shade-grown trees had nearly twice the quantity of taxol (0.076%; $p \leq 0.0001$) as bark from trees exposed to full sunlight (0.042%, Figure 1). Among all 20 trees, concentrations of taxol in the bark were significantly greater than in any of the foliar tissues. Also, in both types of trees, age of the foliar tissues affected the taxol concentrations. For example, in the exposed trees, newer needles and twigs (sampled from the first node at the branch tip) contained the same quantities of taxol (0.011 and 0.010%, respectively; $p = 0.9459$). Older needles sampled from the second node had greater quantities (0.016%; $p = 0.0121$) than younger needles from the first node, indicating that taxol had accumulated, the rate of synthesis had increased, or the rate of catabolism had decreased as the needles aged. Older twigs from the second node had lower ($p = 0.0061$) taxol concentrations (0.007%) than younger twigs (0.010%) as a result of decreased synthesis, increased catabolism, or a dilution effect from the increased production of woody tissue. The latter is a very likely possibility as the older twigs were thicker and more woody in appearance. This is supported by the greater proportion ($p \leq 0.0001$) of woody tissue in the second-node foliage (2.84 g needle/g twig) compared to the first node (3.83 g needle/g twigs). These same relationships were observed for foliage from the shaded trees (Figure 1).

Among all tissues, cephalomannine concentrations were 10.5% to 30.9% of the taxol concentrations. Bark of shade trees contained significantly ($p = 0.0008$) more cephalomannine (0.012%) than the bark of sun trees (0.006%). Among all foliar tissues from shaded and exposed trees cephalomannine concentrations were significantly lower in the twigs than in the needles, except for the first node of shade trees (Figure 2). Needles from exposed trees contained greater quantities of cephalomannine than needles from shade trees, with the greatest differences in the older needles. Among the sun-

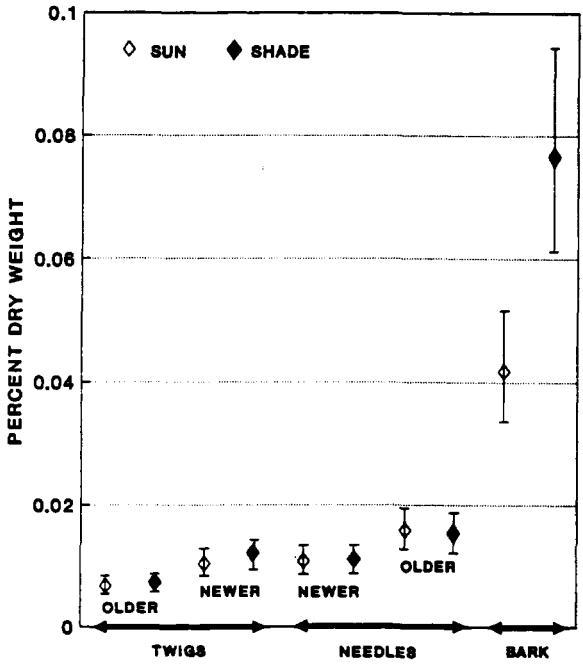

FIGURE 1. Mean taxol concentrations in the tissues of *Taxus brevifolia* exposed to full sun or shade for the previous 6 years. Newer tissue is from the first terminal node and older tissue from the second node. Vertical bars represent 95% confidence intervals.

exposed trees the mean cephalomannine concentration in older needles (0.005%) and bark (0.006%) was not significantly different ($p = 0.2215$). Among the shaded trees, cephalomannine tended to have the same concentration differences as taxol in the various tissues, with greatest concentrations found in the bark. However, among the sun-grown trees the differences in cephalomannine concentrations did not compare with taxol: first, no differences in cephalomannine were detected between the bark and older needles; second, concentrations in the younger twigs were lower than in the younger needles; third, cephalomannine concentrations in older and younger twigs were not detectably different. There are a number of possible explanations, but these data are not adequate to determine which is most probable.

The lower concentrations of taxol and cephalomannine in the bark of sun-exposed trees which have thicker phloem may be the result of a dilution effect. Phloem thickness is related to cellulose and other structural carbohydrates that contribute to dry wt. Assuming other environmental factors at the two sites were similar, trees receiving more light produce more photosynthate and a larger phloem system to transport it. Phloem cells in sun trees may allocate excess photosynthate to cell-wall constituents, or to storage forms of carbohydrates, especially during the late fall and winter. Either of these processes, without a proportional increase in taxane production, would result in decreased concentrations in the bark on a dry wt basis. Although shade trees had 1.8 times greater concentrations of taxol in their bark, less volume of bark was needed from sun-exposed trees to make up an equivalent unit of biomass because its bark was 1.6 times thicker than bark from shaded trees.

In order to maximize the production of taxanes, it will be necessary to understand the various factors that control and influence their biosynthesis, storage, interconver-

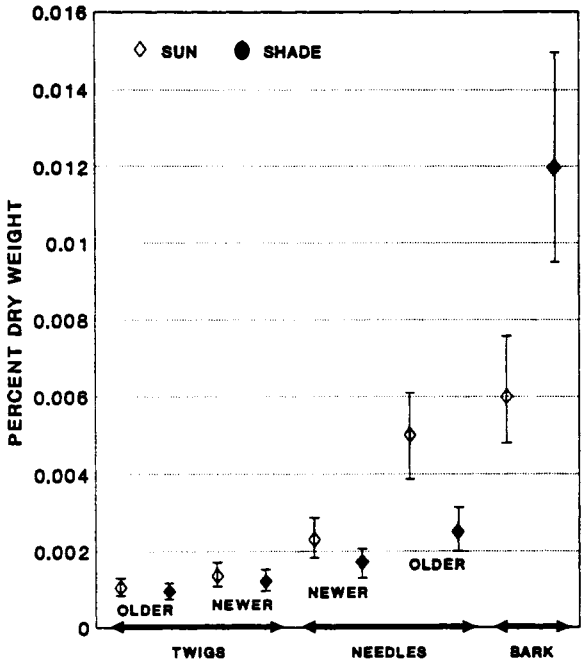

FIGURE 2. Mean cephalomannine concentrations in the tissues of *Taxus brevifolia* exposed to full sun or shade for the previous 6 years. Newer tissue is from the first terminal node and older tissue from the second node. Vertical bars represent 95% confidence intervals.

sions, and catabolism. The degree of genetic control versus the effects of environmental influences on these processes requires further investigation. Genetic variation may account for the variation in bark concentrations of taxol and cephalomannine among individual trees within fairly uniform environmental conditions; e.g., 44.5% and 35.5% coefficients of variation, respectively, among the sun trees, and 38.6% and 59.7%, respectively, among the shade trees.

Harvesting the foliage instead of the bark of Pacific yew for taxol has an advantage over bark in that it is renewable and can be collected yearly without killing the tree. Disadvantages associated with the foliage are lower taxol concentrations, a greater quantity of extraneous material to remove during purification, and the possibility of rapid taxane deterioration after harvesting, although improved harvesting and processing methods might prevent deterioration (2). Taxol concentrations in the foliage did not differ between sun-exposed and shaded trees on a dry wt basis. However, fewer needles from sun-exposed trees make up an equivalent unit of biomass than from shaded trees; thus the foliage from sun-exposed trees would yield a greater amount of taxol on a per-needle basis. Also, needles contained substantially more cephalomannine when exposed to light than when shaded, suggesting that total taxane concentration may be greater in trees receiving more light.

Taxol concentration reported in this paper are 3–10 times higher than those reported by Vidensek *et al.* (7), depending on the tissues being compared. Our taxol concentrations for needle tissue are about twice the amount found by Witherup *et al.* (8). Several factors may contribute to these differences. From our experience the most important are probably post-harvest procedures, storage, and extraction protocol. During

extraction the tissue particle size, type of solvent, solvent temperature, and length of extraction appear to affect the final concentrations. Source of plant material is also important. Even within our restricted populations there was substantial intertree variation, and different geographic sources are likely to have even greater differences. It should also be emphasized that our study was conducted with a restricted population of trees, and the observed responses to light may not be representative of all Pacific yew populations throughout its range.

In summary these data indicate that the individual tree, tissue type and age, and opening of the canopy, can influence taxol and cephalomannine concentrations in samples collected from Pacific yew in late fall. The two compounds did not always vary in the same way within the same tissue and may not be under coordinated control.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The hplc system included a Rheodyne injector with 20 μl sample loop, Perkin-Elmer 410 pump, Perkin-Elmer LC-235 diode array detector, and an Epson Equity 1+ computer with Omega software for data collection and integration. Separations were achieved with an Alltech Lichrosorb RP-18 guard column and column. The column, 250 × 4.6 mm, 5 μm particle size, had been previously end-capped with Alltech hplc column end capping agent. For analysis, 60 μl of extract was injected through the sample loop. The solvent system was MeOH-H_2O (65:35) (7), 1 ml/min, isocratic for 30 min, detection 225 nm. To improve quantitation the column was flushed between samples with a linear gradient replacing H_2O with MeCN over 7 min, then back to 35% H_2O with another 7 min gradient. The isocratic mobile phase was equilibrated 11 min before injecting another sample. Standard curves were prepared with cephalomannine (Rt = 22.9 min) and taxol (Rt = 25.4 min) supplied by the National Cancer Institute, and used for quantitation by the external standard method. These two compounds were baseline-resolved in all extracts. Solvents were hplc grade, except the H_2O which was obtained from a NANOpure water deionizing system attached to the distilled H_2O supply.

PLANT MATERIALS.—Twenty Pacific yew trees, identified by the authors, were selected and tagged for study on the Oregon State University, College of Forestry, McDonald Research Forest. These trees are part of a continuing study; herbarium specimens are deposited with the authors. Ten of the trees (7 males and 3 females) were growing in the shade of a forest overstory (210 m elevation), and less than 1 km away another ten (5 males and 5 females) were growing in full sunlight in a 5-hectare area where the overstory had been removed (305 m elevation). The exposed trees had been growing in full sun since 1984. Irradiance was measured during peak photosynthetic activity on two successive sunny days with a Li-Cor 190s quantum sensor. Diameters of trees were measured as well as sapwood thickness taken from cores. From 11:00 am to 4:30 pm, December 6, 1990, foliage and bark samples were collected from each tree. Air temperatures during the collection period were cool, between 2° and 7°. Two branches were clipped from each of the north and south sides of each tree and stored in plastic bags for transport to the laboratory. Two rectangular samples of bark (approximately 5–6 × 8–9 cm) were collected from breast height on the north and south sides of each tree. They were combined and sealed in a ziplocking plastic bag and placed on ice for transport.

In the laboratory the bark was immediately placed in a 50° oven and dried for 24 h. The dried bark was sealed within two ziplocking plastic bags and stored in a −26° freezer. Larger bags containing branches were immediately placed in a walk-in cooler (3–4°) and stored 96 h while the foliage was sorted; needles were left attached to the twigs. Diseased or abnormal twigs were discarded. These tissues were oven-dried 24 h at 50°. Dried samples were kept at room temperature for 6 days while the needles were carefully separated from the twigs and all buds removed. After each needle and twig sample was weighed, it was stored at −26° as described for the bark. Just prior to analysis the tissues were ground in a Wiley Mill to pass a 20 mesh screen, then redried in vials at 50° for 1 h, sealed, and cooled to room temperature before weighing.

In the initial analysis of these data (9), needles taken from the first node were identified as current year, or 1990, and needles from the second node as past year, or 1989. But more extensive observations of the morphology and ontogeny of the foliage indicated these distinctions were not always precise. Therefore, the foliage is described here with confidence as younger first node and older second node tissue.

Subsamples of fresh foliage were removed from the larger bags, obvious current-year needles were randomly selected, and 20 fully expanded needles removed for the measurement of leaf area and dry wt to calculate leaf area ratios (6). Projected leaf area was measured with a LiCor surface area meter. Surface area was multiplied by two and divided by dry wt to calculate specific leaf area. Bark thickness was measured with a digital micrometer on four sides of two bark samples taken from each tree, as described above, on June 3, 1991.

EXTRACTION.—Approximately 2 g of dried ground tissue was weighed into a 50-ml Erlenmeyer flask, covered with 20 ml MeOH, and sealed. The solution was shaken for 16 h at 3–4° and pipetted into a sealed centrifuge tube. The cold extraction was repeated for 24 h on the shaker with an additional 20 ml of MeOH. The two solutions were thoroughly mixed and centrifuged to remove particulates. A 20-ml aliquot was evaporated to dryness with vacuum and a 50° H_2O bath. The residue was dissolved in 10 ml H_2O, followed by 10 ml CH_2Cl_2, and swirled vigorously. The solution was transferred to a separatory funnel made from a 20-ml plastic syringe fitted with a stopcock. After sealing, the funnel was centrifuged for 5 min to break up the residual emulsion. Part of the recovered CH_2Cl_2 layer (5 ml) was evaporated to dryness with vacuum at room temperature. The residue was redissolved in MeOH (1.0 ml for needles and twigs, 2.0 ml for bark), filtered through a Nylon 66 membrane (0.2 μ), and analyzed by hplc.

STATISTICAL ANALYSIS.—The experiment was designed as a split-plot, where the trees were the experimental units of the whole plot and the various tissues were the experimental units of the subplot. Taxol and cephalomannine concentrations were separately analyzed by the General Linear Models Procedure using the SAS Institute (10) statistical program. Prior to analysis each data set was transformed to their natural logs to eliminate heteroscedacity. Significantly different means were identified by Fischer's (protected) least significant difference (LSD, $\alpha = 0.05$). All reported means are the original observed data. Confidence intervals (95%) in Figures 1 and 2 were obtained by back-transforming the natural log means after adding or subtracting $t_{0.025,72}$ (ca. 2.0), the least squares mean standard error. Bark thickness, sapwood ratios, leaf morphology, and stem diameters of exposed and shaded trees were separately analyzed by a one-way analysis of variance using the Statgraphics statistical program (11).

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