'HICKSII' YEWS AS A SUSTAINABLE SOURCE OF ANTICANCER COMPOUNDS

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Introduction

Taxol® is an effective anticancer drug identified in the 1960s. Taxol® is Bristol-Myers Squibb's registered trademark for paclitaxel. Paclitaxel is commonly referred to as 'Taxol' in the press and coversation, the term will be used here. Taxol has been refined from the bark of the Pacific yew tree, *Taxus brevifolia* Nutt. This slow-growing tree is native to the temperate northwestern United States and adjacent Canada ranging from southern Alaska to northern California and east to Montana. Taxol is also found in small twigs, roots, and needles of *T. brevifolia* and several other *Taxus* species. A number of these species, including *T. x media* 'Hicksii', commercially available from nurseries, grow faster than the native species and produce useful amounts of Taxol.

Other compounds with a complex ring structure like the one in Taxol, collectively known as "taxanes", are found in many yew species. Although other taxanes do not generally have anticancer activity, the other taxanes may be used for synthesis of Taxol.

Taxanes, like many other secondary plant products, may be synthesized in response to environmental or biotic stress. Environmental stresses such as ultraviolet light, heat, or drought may therefore enhance taxane biosynthesis. Stressful environmental conditions increase hormones such as abscisic acid (ABA) which affect root growth and plant survival under water-stressed conditions.

Landscape yews thrive in the Snake River plain of eastern Oregon and southwestern Idaho, producing large masses of annual growth. The major objective of this project was to study the effects of water stress on the amount of Taxol or other taxanes and ABA made by the landscape yew, *T. x media* 'Hicksii', a cultivar with high Taxol content. Water stress could be monitored and controlled effectively in the dry desert climate at the Malheur Experiment Station in Ontario, OR. The shrubs could be managed commercially for repeated harvesting in hedge rows. Shrubs were supplied with controlled amounts of water from June through August of 1996 and 1997. The amount of taxanes extracted from the experimental shrubs receiving each of three irrigation treatments was compared. Soil and plant water potentials and the degree of stomatal closure were compared, and changes in the concentration of ABA were determined.

Materials and Methods

Plant material, soil and irrigation system. Cutting-propagated yew shrubs (*Taxus x media* 'Hicksii' Rehd.), approximately 2 feet tall, were obtained from Zelenka Nursery Inc. (Grand Haven, MI). They were planted on May 3, 1996 in an Owyhee silt loam at the Malheur Experiment Station. The shrubs were planted 4 feet apart in rows 10 feet apart with the idea that they could eventually grow into hedge rows.

Initial soil analysis in the spring of 1996 showed pH 8.0, 2.6 percent organic matter, 6 ppm nitrate-N, and 20 ppm ammonium-N. Other soil extractable soil nutrients included 924 ppm K, 4920 ppm Ca, 402 ppm Mg, 336 ppm Na, 1.8 ppm Zn, 11.5 ppm Fe, 7.4 ppm Mn, 2 ppm Cu, 14 ppm sulfate-S, and 0.7 ppm borate-B. The field received a banded fertilizer mix on June 17, 1996, consisting of 100 lb N/acre as urea, 100 lb P_2O_5/acre as triple superphosphate, 100 lb S/acre as elemental sulfur, and 1 lb Zn/acre as zinc sulfate.

The shrubs were irrigated uniformly for two months after planting using furrow irrigation, after which they were subjected to one of three irrigation rates using drip irrigation. Starting July 15, 1996, one drip tape (Turbulent Twin Flow, Chapin Watermatics, Watertown, NY) was used along each row to irrigate the shrubs. The drip tape had emitters spaced one foot apart and a flow rate of 0.5 gpm/100 ft of tape at 10 psi water pressure. The irrigation treatment rates were 1.1 acre-inch/acre (minimally stressed, full rate), 0.77 acre-inch/acre (moderately stressed, 70 percent of 1.1 acre-inch/acre), and 0.44 acre-inch/acre (stressed, 40 percent of 1.1 acre-inch/acre) of water was applied at each irrigation. The water application rate was calculated based on a 3.3-foot wide bed for each shrub row. Irrigation rates were applied to plots one row wide and five shrubs long arranged in a complete randomized block design with four replicates each. Total applied water was measured for each irrigation treatment using water meters.

Soil water potential. All plots were irrigated when the average soil water potential (SWP) at a 8-inch depth in the minimally stressed plots reached -40 kPa in 1996 and -60 kPa in 1997. Two granular matrix sensors (GMS, Watermark Soil Moisture Sensor Model 200SS, Irrometer Co., Riverside, CA) were installed at a 8-inch depth in line with the shrubs in each plot. The GMS were previously calibrated to measure SWP. All GMS were connected via multiplexers (AM 410 multiplexer, Campbell Scientific, Logan, UT) to a datalogger (CR 10 datalogger, Campbell Scientific, Logan, UT. The SWP was recorded daily at 8 AM from July 18 to August 28 in 1996 and from June 3 to August 28 in 1997.

The shrubs were irrigated according to the treatment criteria on July 15, August 8, and August 22, 1996, and on June 25, July 11, July 28, August 9, and August 24, 1997. Based on 3.3-feet wide beds, a total of 5.27, 4.28, and 3.32 acre-inch/acre in 1996, and 9.37, 7.58, and 6.08 acre-inch/acre in 1997 were applied to the 1.1, 0.77 and 0.44 acre-inch/acre treatments, respectively. These amounts of water include 2 acre-inch/acre applied to all treatments after the last sampling date each year and 2 acre-inch/acre applied uniformly during May 1997.

Harvesting methods and schedules. A sample handling procedure was needed that would stop biosynthesis and degradation of taxanes and ABA at harvest. To determine how to handle harvested samples, six composite samples were randomly taken from plants in each of the plots on June 30, 1996, placed in 72 plastic zipper bags and assigned to one of three treatments: oven dried at 100 ^{0}F or storage in coolers with either liquid nitrogen or dry ice.

Each summer, twigs with leaves and stems (6 to 8-cm lengths) were taken from each plot individually before the onset of stress and approximately every three weeks during the remainder of the irrigation season. Samples were taken from each of the middle three shrubs in each plot of five shrubs four times during the summer of 1996 and six times during the summer of 1997. All samples were frozen immediately and transported overnight on dry ice to the University of Portland for analysis. If not analyzed upon arrival, they were stored at -80 ^{0}C.

Soil water potential and stomatal closure. Twigs were sampled each day during an irrigation cycle from July 14 to July 29, 1997, to determine the relationship between plant water potential and SWP. Plant water potential was measured using a Scholander pressure chamber (PMS Instruments Company, Corvallis, OR). Chamber pressure was recorded at the point at which xylem exudate appeared on the cut end. As the cuttings were collected for plant water potential analysis, the backs of some needles on shoots of the same plants were painted with clear fingernail polish. These shoots were immediately cut and packed on dry ice and sent to the University of Portland. Upon arrival in Portland, the polish was peeled off and prepared for viewing with a light microscope. The polish bears the imprint of the stomata.

Taxol and abscisic acid determination. Taxol and other taxanes were analyzed as described by Hoffman et al. (1996).

Results and Discussion

Establishment of handling procedures. Three protocols were tested to find the most reliable method for harvesting and transporting samples from Ontario, OR to Portland, OR. Samples stored in liquid nitrogen or on dry ice had higher Taxol content than those that were oven dried. Some of the oven-dried cuttings yielded little or no Taxol. All subsequent samples were collected and immediately placed in a cooler with dry ice and transported overnight in the cooler for analysis at the University of Portland.

Responses to water stress. Tissue samples were harvested and analyzed four times during the summer of 1996 and six times during the summer of 1997. The samples were generally taken just before irrigation when SWP was lowest and moisture stress was highest. There were several important differences between the field conditions in 1996 and 1997. First of all, the plants had better established root systems in 1997, so the data were probably more reliable and consistent. Secondly, the SWP that was set as an irrigation criterion in 1997 was -60 kPa, drier than the -40 kPa used in 1996, the year of shrub establishment. There was a greater difference between SWP in stressed and minimally stressed plots in 1997. This difference was apparently large enough to effect changes in plant ABA and Taxol content.

During the irrigation cycle between July 14 and 28, 1997, both SWP and plant water potential were measured and recorded daily. There was a strong positive correlation between SWP and plant water potential. Plants with more negative SWP also tended to have more negative stem (xylem) water potential. This correlation suggests that measurement of SWP may be used to approximate the amount of water stress within the plants.

On July 28, 1997, when the samples were harvested for taxane and ABA extraction, needles were also collected for stomatal analysis. Only a few stomata were open on needles from any of the plants, and none were open on those that were most stressed. Stomata from all the leaves were essentially the same size. The ABA concentration tended to be higher in shoots from plants grown on plots with more negative SWP. These were also the plants with the fewest open stomata. There were strong correlations between ABA concentration, SWP, and the percentage of open stomata.

Taxane and ABA analysis. Taxanes and ABA were extracted and quantified periodically throughout the summers of 1996 and 1997. In 1996, the plants were beginning to establish themselves in the soil. Differences between the amounts of Taxol recovered changed throughout the summer. By the end of the experimental time, significantly more Taxol was obtained from moderately water-stressed plots as compared with the other treatments. The range of Taxol recovery was broad, possibly due to seasonal and weather differences, plant-to-plant variation, and the fact that root systems were still becoming established during the period of treatment.

After the plants were better established, the 1997 irrigation criterion was lowered to -60 kPa. There was still variation in the amount of Taxol recovered from the experimental plots throughout the summer, but the pattern of recovery had changed . The overall trend for 1997, however, is clear: both the taxanes and ABA concentrations were higher in the stressed plants than in the plants receiving more water.

Strategy for hedge-row yew harvesting for increased Taxol production. To our knowledge, the present study is the first to relate soil and plant water potential with taxane recovery from yew. The strong correlation between the Taxol and ABA recovered from yew suggests that either the conditions that increase ABA also increase Taxol or that when ABA concentrations rise, they may somehow lead to the increase in Taxol. If yew were grown as a crop, it would be important to monitor field conditions and taxane content so as to determine the best time for harvesting clippings. It is more practical to routinely monitor the SWP than to measure plant water potential or ABA concentrations in the tissues.

There is a strong correlation between taxane concentration and SWP treatment. As the SWP decreases, the concentration of Taxol increases. Further studies may be able to fine-tune the timing and amounts of irrigation required to keep the plant growing vigorously and also harvest high yields of Taxol per gram of plant material. A SWP of about -70 kPa could be maintained before harvesting. The trees have one growth flush in the spring, and the effects of water stress subsequent to spring growth on subsequent growth of yew shrubs is not known. The length of time necessary to hold the plants at water-stressed conditions to induce increased Taxol synthesis is also unknown.

Conclusion

Taxol® (paclitaxel), an important anticancer agent, is found in many species of yew. As the need for Taxol increases, it will be necessary to find sustainable sources for this drug. We have shown that *T. x media* 'Hicksii' shrubs can be grown in the dry climate of Ontario, OR. Hedge-row harvesting of the shrubs may be planned to optimize Taxol production by monitoring SWP. Under the conditions described here, the stressed shrubs produce about 50 percent more Taxol than minimally stressed shrubs. Branches could be harvested annually at predetermined conditions through hedge-row clipping to produce a sustainable supply of Taxol and other taxanes from which Taxol or similar compounds may be synthesized.

Literature Cited

Hoffman, A.M., C.C.J. Voelker, A.T. Franzen, K.S. Shiotani, J.S. Sandhu. 1996. Taxanes exported from *Taxus x media* Hicksii cuttings into liquid medium over time. Phytochemistry 43:95-98.