Taxol[1] Production in Nodule Cultures of *Taxus*

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The in vitro synthesis of secondary compounds from plants is one source of scarce and valuable phytopharmaceuticals. Often, some level of cellular or tissue differentiation is needed for the biosynthesis of many of these important compounds. Nodule cultures, consisting of cohesive multicellular units displaying a high degree of differentiation, were initiated from cultured needles of seven *Taxus* cultivars (*Taxus cuspidata, Taxus x media* 'Hicksii', *Taxus x hunnewelliana* 'Richard Horsey', *Taxus x media* 'Dark Green Spreader', *Taxus x media* 'L. C. Bobbick', and *Taxus brevifolia*). Under normal semicontinuous perfusion culture conditions (bimonthly refreshments to yield 0.2% sucrose), only trace amounts of taxol were detected from *Taxus* nodule cultures. However, with an elevated sucrose level (0.5% or 1.0%), taxol production was enhanced in *T. cuspidata* nodules to approximately 12 μg taxol/g nodule dry weight (dw). Stimulation of taxol production by elevated sucrose levels occurred even in the absence of other nutrients. The effect of increased sucrose on taxol induction does not appear to be due to an osmotic effect in the medium, suggesting that the increase in taxol production may be correlated with a metabolic process within the nodules. Although sucrose had a significant effect on taxol production, taxane precursors or elicitors of terpenes, as well as other plant secondary metabolites, had no effect on the production of taxol from these cultures. In addition to taxol, the higher sucrose levels also induced the production of 7-*epi*-10-deacetyltaxol, cephalomannine, and 7-*epi*-10-deacetylcephalomannine, so that total content of these taxanes equaled approximately 39 μg taxane/g dw nodules.

Taxol, a diterpene obtained from *Taxus* spp., has significant anticancer activity[3,4] and is clinically effective both alone and when employed with other cancer therapy treatments.[4] One important and interesting property of taxol is its novel mechanism of action. Taxol polymerizes and stabilizes microtubules.[5,6] This is in contrast to the mechanism of other phytochemical mitotic blocks, such as the vinca alkaloids, that *de*stabilize microtubules.[6,7]

Until recently, the bark from the Pacific yew *(Taxus brevifolia)* was the only commercial source of the drug. Alternative sources for taxol include harvesting horticultural *Taxus* species, semisynthesis of taxol from taxane precursors, total chemical synthesis of taxol, and in vitro production of taxol by cell or tissue culture. Each of these alternative sources offers unique advantages. Still no one method presently appears to be superior in satisfying the long-term demand for taxol.

Numerous groups have reported the production of taxol and related taxanes from various types of cultured tissues.[8−14] However, there have been no published reports of the commercial scale production of taxol from microculture. In fact, commercial production levels have been achieved in plant bioreactors with very few plant secondary compounds. One possible reason is that most of these tissue culture systems lack significant cellular organization or tissue differentiation for production of either the desired compound or its precursors. These features may be required for the compartmentalization of steps in the biosynthetic pathway needed for the complete synthesis of the desired compound. Also, the secondary compound may be toxic to the cells, in which case cell growth and product synthesis would be negatively correlated.

For these and other reasons, plant cell cultures often produce secondary metabolites in only a fraction of the amount produced in vivo.[15,16] Despite these limitations, some cell cultures can be manipulated for increased metabolite production through the use of elicitors,[17−19] media modifications,[14,20,21] and biotransformation with the use of metabolic precursors.[22−24]

The production of ***nodules***, spherical, cohesive cellular units displaying consistent internal structure,[25] may provide a culture system well suited for the production of taxol and other valuable phytopharmaceutical products. Nodules consist of cells capable of dividing on the exterior, providing increases in biomass, and on the interior, an extent of differentiation which may be optimal for either production or sequestration of secondary metabolites.[15,26,27]

In this paper, we report the induction of nodules from six *Taxus* cultivars and sustained production of taxol using a semicontinuous perfusion culture. Several strategies for taxol production from *T. cuspidata* nodules were employed, including manipulation of nutrient and sucrose levels and the use of elicitors and biochemical precursors.

Results and Discussion

When cultured on nodule induction medium, needles excised from *Taxus* shoot cultures exhibit cellular proliferation at the cut ends. The cell masses subsequently differentiate into nodular tissue. After 4−6 weeks, callus and nodule primordia can be visually seen, and after 12 weeks, discrete masses of cellular aggregates form at the cut end of the needles. These aggregates contain multiple well-formed nodules (Figure 1) which become independent units upon excision from

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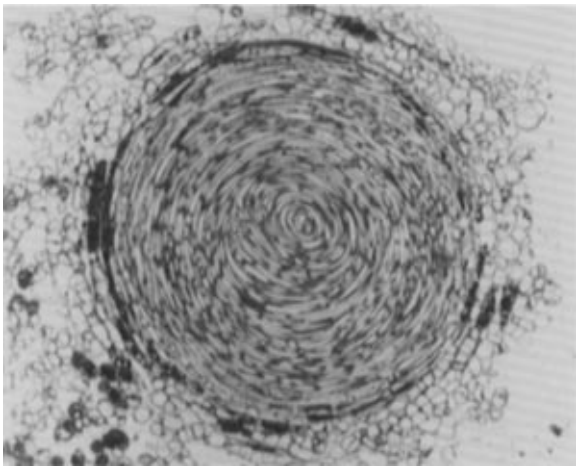

Figure 1. Cross-section through a mature *T. cuspidata* nodule. The nodule was fixed in 2.5% glutaraldehyde and 2.5% *p*-formaldehyde, dehydrated in a graded ethanol series, and embedded in L. R. White resin, and 1 μm sections were taken.[30] The sections were viewed and photographed with a Zeiss Ultraphot II light microscope.

the needles and subculturing. Both auxin and cytokinin are required for efficient nodule induction. In the absence of hormones or when either cytokinin or auxin is used alone, no nodular tissue forms. In contrast, up to 80% of the excised needles can be induced to form nodular tissue with both cytokinin and auxin present in the medium.

Mature *Taxus* nodules are spherical cellular aggregates composed of an interior xylem region, a vascular cambium, and an outer phloem region. In median-sectional view, well-developed *Taxus* nodules have a central region of secondary xylem composed of tracheids and uni- and biseriate rays (Figure 1). The tracheids are spirally arranged with a spherical vascular cambium completely ensheathing the xylem. In turn, the cambium is completely surrounded by secondary phloem. *Taxus* nodules are similar in morphology to previously described poplar nodules.[25]

Under the subculture regime described, long-term culture and morphological integrity of the nodules can be maintained. This bimonthly refreshment regime was developed after extensive trials with different nutrient and hormone levels, as well as other subculture and refreshment regimes (data not shown).

When subcultured to undiluted medium, brown discoloration of the medium and some browning of the tissue occurs during the first 2 weeks. These effects eventually lead to deterioration of the culture lines. Ethylene evolution in nodule cultures is initially high (16.7 nL/h/g fresh weight (fw) 2 days after transfer of the nodules) and remains relatively high even after 11 days (6.5 nL/h/g fw) on undiluted media. With dilute media, a far greater drop in ethylene evolution occurs (from 13.0 nL/h/g fw after 2 days to 0.6 nL/h/g fw after 11 days). In addition, the brown discoloration is less severe and of a shorter duration. These results suggest that the shock of subculture is minimized with the use of dilute media.

When subcultured to dilute media, growth ceases unless the cultures are periodically refreshed (semicontinuous perfusion). The refreshment regime employed maintains healthy green nodule cultures. Hormone levels are also important to the maintenance of stable nodule cultures. At lower cytokinin levels, nodular organization is not as well defined and the proliferation of less differentiated callus is favored. At higher auxin levels, both callus proliferation and browning of the nodules occurs.

Currently, nodule cultures have been induced from *Taxus cuspidata, Taxus x media* 'Hicksii', *Taxus x hunnewelliana* 'Richard Horsey', *Taxus x media* 'Dark Green Spreader', *Taxus x media* 'L. C. Bobbick', and *T. brevifolia* shoot cultures. However, because *T. cuspidata* responded well in vivo and was the focus of other research, all experiments reported in this paper utilized *T. cuspidata* cultures.

Nodules grown on the standard 0.2% sucrose regime with bimonthly refreshments (exchanging one tenth of the culture medium for fresh medium with 2% sucrose for each refreshment) generally contain either no detectable taxol or a very low level (Table 1). Taxol has never been detected in the medium.

In subsequent experiments, different sucrose concentrations in refreshments containing either medium or water (Table 1) showed that sucrose has a positive effect on taxol production from nodules, even in the absence of nutrient supplements. Note that all sucrose concentrations listed below are the concentrations achieved just after refreshment. Nodules grown on all sucrose levels in water show a slight taxol induction. In contrast, cultures grown on 0.5% and 1.0% sucrose with dilute medium show significantly higher levels of taxol in the nodules (mean of 9.1 μg taxol/g and 10.9 μg taxol/g dw, respectively. These taxol levels are equivalent to 253 μg taxol per 1 L bottle or 100 mL medium. Taxol is rapidly induced from these cultures after only 6 weeks, and the production levels are maintained throughout the 12 week culture period.

In addition to taxol, 7-*epi*-10-deacetyltaxol cephalomannine and 7-*epi*-10-deacetylcephalomannine (Table 1) are also induced by a higher sucrose level. The levels of 7-*epi*-10-deacetyltaxol are comparable in both the medium and the water refreshment regimes with 0.5% sucrose inducing the highest levels (5.2 μg taxol/g dw). Both cephalomannine and 7-*epi*-10-deacetylcephalomannine are only quantifiable at 12 weeks; clear UV absorption spectra to confirm identity were difficult to obtain. The induction of cephalomannine requires the presence of medium as it was not induced in the water controls. In contrast, 7-*epi*-10-deacetylcephalomannine was induced at the higher sucrose levels at equal levels in water or in medium. The production of baccatin and 10-deacetylbaccatin could not be confirmed due to the occurrence of unknown compounds which coeluted with baccatin and 10-deacetylbaccatin.

The levels of taxol induced in the nodule cultures (approximately 0.001%) are substantially lower than the generally referenced 100 μg/g dw (0.01%) taxol found in the bark of *T. brevifolia*[28] and also lower than the level found in *T. cuspidata* needles and shoots.[10,29] Although direct comparison of taxol levels between tissue culture systems is difficult due to nonuniform methods of determining taxol concentrations, the levels of taxol detected in nodule cultures are generally less than from other in vitro systems. For example, taxol levels from *T. cuspidata* callus, suspension cultures, and immobilized cells were 0.02%, 0.0014%, and 0.012% respectively,[9] 0.05% from *T. baccata* suspension cells,[19]

Table 1. Taxol Concentration [(μg Taxane/g dw (±SE)] from *T. cuspidata* Nodules Cultured for Varying Time Periods under Different Nutrient and Sugar Regimes[a]

		refreshment medium			water		
taxane	weeks	0.2%[b]	0.5%	1.0%	0.2%	0.5%	1.0%
taxol	6	0.0	6.8 (3.2)	8.6 (2.1)	0.0	3.7 (1.2)	1.7 (1.1)
	10	0.2 (0.1)	7.1 (1.5)	2.1 (5.9)	0.8 (0.8)	1.5 (1.3)	1.3 (0.6)
	12	0.2 (0.1)	9.1 (1.5	10.9 (4.5)	1.3 (0.9)	0.2 (0.2)	0.0
7-*epi*-10-deacetyltaxol	6	0.0	1.5 (1.5)	0.0	0.0	1.0 (1.0)	1.0 (1.0)
	10	0.0	3.4 (3.4)	3.5 (2.7)	0.0	2.7 (2.6)	1.1 (1.1)
	12	0.0	5.2 (3.6)	0.0	1.0 (1.0)	4.5 (3.5)	2.5(1.3)
cephalomannine	12	0.0	11.8 (4.1)	13.3 (9.8)	0.0	0.0	0.0
7-*epi*-10-deacetylcephalomannine	12	0.0	6.4 (3.2)	4.6 (2.9)	0.0	7.0 (3.9)	5.5 (2.7)

a All cultures were initially maintained on dilute medium with 2% sucrose prior to the initiation of the experiment. *b* Percent sucrose in medium immediately after refreshment.

and 0.006% from *T. cuspidata* embryogenic cultures (Wann, S. Personal communication).

In addition to taxane production, cultures refreshed with higher sucrose concentrations also have increased fresh and dry weights compared to 0.2% sucrose. This increase is more apparent in nodules refreshed with medium than in the water treatment (data not shown).

The nodules grown on 1.0% sucrose (both water and medium) exhibit both browning and senescence at the end of 12 weeks. Such nodules are soft in texture and slough off cellular debris. Therefore, this treatment was terminated after taxane profiles were analyzed. It is interesting to note the sensitivity of nodule cultures to sucrose, as the levels used for these experiments are 2−10× lower than those conventionally used for other microculture systems.

Taxol levels are relatively stable in nodule cultures remaining on 0.5% sucrose over the 12 week experimental period. As expected, nodules subcultured from 0.2% to 0.5% sucrose show an increase in taxol levels. Cultures refreshed *weekly* at 0.5% sucrose with only half the nutrient and hormone complement in the medium develop similar senescence as nodules refreshed *bimonthly* on 1.0% sucrose/full medium. This treatment was designed to recreate the levels of nutrients and sucrose added to the highest taxol-producing cultures (1.0% sucrose/full medium) every 2 weeks, but to halve the amount added per weekly refreshment. Unfortunately, this approach is just as stressful to the cultures as the 1.0% sucrose refreshment regime every 2 weeks.

The stress of higher sucrose levels on the nodule cultures is apparent. Even when refreshed with 0.5% sucrose, the nodules eventually show signs of distress (browning, sloughing of outer cells, etc.). In contrast, cultures kept on 0.2% sucrose remained unaffected, yet do not reliably produce taxol. This detrimental response and the increased taxol levels could be related to the carbon source or to the osmotic stress caused by higher solute concentration in the culture medium. Therefore, osmotic concentrations matching those of the elevated sucrose were tested using sugars which are not readily taken up by plant cells (mannitol and sorbitol). Osmotic levels were adjusted as a refreshment treatment of equal parts mannitol and sorbitol to medium already containing 0.2% sucrose, to simulate the osmolarity of medium containing 0.5% and 1.0% sucrose. Cultures exposed to media with higher osmotica exhibit signs of stress very early, in that the medium turns brown or red within 24 h after addition of the first refreshment. This secretion of putative phenolic compounds worsens considerably over the following 2 weeks, almost to the point of opacity. In addition, cultures exposed to the osmotically adjusted medium show a general trend of higher pH and conductivity compared with the 0.2% or 0.5% sucrose controls. These results indicate ion and particulate leakage into the medium, suggesting cell senescence. Along with the pH and conductivity changes in the medium, the nodules treated with osmotic refreshments also show signs of stress such as browning, softening of tissue, and accumulation of particulate matter in the medium. In contrast, controls on 0.5% sucrose appeared to be healthy and stable, although these eventually succumbed to browning as well. At 3 weeks, no taxol was detected from the high osmotic treatments.

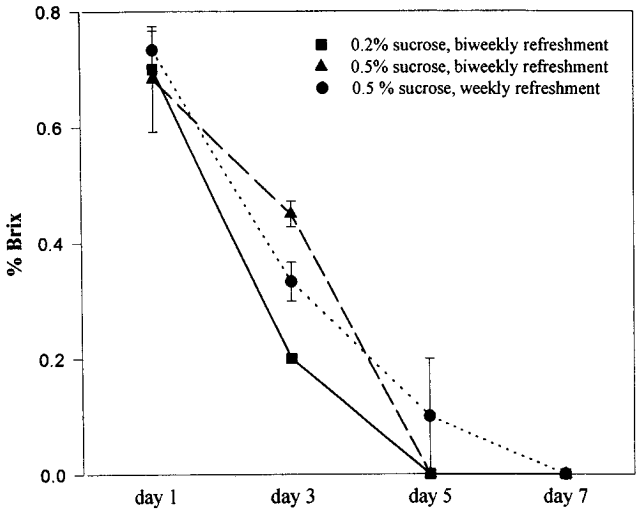

Figure 2. Relative sucrose content of *T. cuspidata* nodule culture medium after different time periods following refreshment with refreshment medium containing various sucrose levels, as measured by a hand refractometer and expressed as % Brix. Vertical bars represent SE.

Since the osmotic concentration in the medium did not appear to account for the increased taxol content at higher sucrose levels, measurements of percent Brix were taken to give an indication of the absorption of sugar by the nodule cultures. The percent Brix of the culture medium is reduced to near zero 5 days after refreshment (Figure 2). In fact, within 3 days, a significant decrease in percent Brix can be observed. This indicates that the sucrose in the medium is very rapidly taken up or metabolized by nodule cultures.

The decline of the nodule cultures at high sucrose levels may be related to the simultaneous accumulation of higher levels of taxanes. However, several lines of evidence (data not shown) do not support this hypoth-

esis. *Taxus* nodule cultures spiked with high concentrations of taxol (20 μM) continue to grow even though up to 90% of the taxol is found in the nodules. In addition, recent work by Russin et al.[30] indicates that taxol accumulation may be localized to *Taxus* cell walls and not the cytoplasm, thus sequestering it away from the microtubules inside the *Taxus* cell.

Manipulations of the nodule cultures other than elevating sucrose levels, such as feeding precursors and elicitors, induced no detectable taxol production. Both phenylalanine and acetate are taken up by *Taxus* shoot cultures (Ellis, D. D. Unpublished results) and have been incorporated into taxol.[23] Phenylalanine has also been reported to increase taxol production in *Taxus* suspension cultures.[24] Methyl jasmonate was chosen as a potential inducing agent because of its role in the elicitation of defense compounds.[31] No apparent stress was induced in the cultures by either phenylalanine or methyl jasmonate, but potassium acetate at 100 mM caused a response similar to that evoked by high osmotic medium.

Other manipulations done to the nodule cultures included comparison of parallel cultures grown in light and dark conditions and the inoculation of nodule cultures with *Leptographium terebrantis*, a fungus known to induce monoterpenes in pine.[32] Neither of these treatments induced taxol production in the nodule cultures. Both inoculation with the fungus and inoculation with spent fungal medium caused rapid nodule death.

Nodule cultures of *Taxus* species can be generated, continuously subcultured, and induced to form taxanes, including taxol. Although these nodule cultures appear to be highly differentiated, levels of taxane production, at least under the conditions tested, are not superior to other in vitro culture systems. However, the high degree of cellular and tissue differentiation in nodule cultures provides a tool which might be useful for the production of taxanes other than taxol as well as secondary metabolites from other plant species.

Experimental Section

Nodule Culture Initiation. The reliable induction of nodules depends on the establishment of stable shoot cultures from field-grown *Taxus* plants.[10] Nodules were induced on leaves (needles) excised from healthy, succulent shoot cultures. Fifty excised needles were placed in a 1 L bottle (Corning #1395) containing 50 mL of *nodule induction medium* consisting of a modified WPM.[33] The modifications included the deletion of K_2SO_4 and glycine, the addition of 1150 mg/L KNO_3, and an extra 1500 mg/L $CaNO_3$, as well as the use of additional vitamins including 3 mg/L thiamine HCl, 0.1 mg/L riboflavin, and 1 mg/L each of nicotinic acid, pyridoxine HCl, biotin, choline chloride, folic acid, and D-pantothenic acid. This medium was supplemented with 2% sucrose, 10 μM NAA, and 2 μM 2-((γ-γ-dimethylallyl)amino)purine. The culture bottles were loosely covered with foil-covered caps, turned 1/4 turn back from tight to allow gas exchange, and incubated horizontally on a roller bottle apparatus (Wheaton #348940) at 1/2 rpm (bottle speed) under constant cool white fluorescent illumination at 25 °C.

Cellular aggregates at the cut end of the needles were carefully excised from the needle after 12 weeks and cultured in 100 mL dilute nodule induction medium under the above conditions. The dilution for this initial nodule culture was done by bringing the original 50 mL induction medium up to 90mL with sterile distilled H_2O and adding 10 mL of fresh medium supplemented with 2% sucrose, 1 μM NAA, and 50 μM zeatin (hereafter referred to as *refreshment medium*). The nodule cultures were refreshed bimonthly by replacing 10 mL of spent medium with the same amount of refreshment medium. With this culture regime, the final sucrose concentration in the medium immediately following refreshment was 0.2%.

Nodule cultures were subcultured every 8 weeks by placing 5 g of screened (>2 mm in diameter) nodule tissue into each new culture, using diluted medium and culture conditions as above.

Taxol Extraction. One gram of blotted-dry weight of nodules from established nodule cultures was ground in 6 mL of 95% ethanol and filtered through a Millipore AP25 borosilicate microfiber glass prefilter. This ethanol extract was diluted 10-fold with dH_2O, and loaded onto a solid-phase extraction cartridge (Waters C_{18} Sep-Pak). Prior to use, the Sep-Pak was washed with 100% acetonitrile (AcCN) and equilibrated with 9.5% ethanol. After the sample was loaded the Sep-Pak cartridge was rinsed with 10 mL of 9.5% ethanol. Taxol was eluted with 75% AcCN. The eluate was diluted to 60% AcCN with dH_2O and filtered through a 0.2 μm syringe filter (Gelman Acrodisk 13CR PTFE). The resulting sample was analyzed directly by HPLC (Hewlett-Packard 1090 LC, 339 integrator, and 1040A diode-array detector monitoring absorbance at 228 nm) using a Metachem Hypersil 3 μm C_{18} 50 × 4.6 mm column, with an injection volume of 25 μL. Samples were eluted from the column in an AcCN:water gradient, 30:70 to 50:50, over 10 min with a flow rate of 1 mL/min. Identification of the taxane fraction was confirmed by coelution and comparison of UV absorption spectra with a taxane standard. In addition, identification of the taxol fraction was confirmed by mass spectroscopy. Quantification was done by comparison to taxane standard curves. All samples were processed fresh without drying and were analyzed as quickly as possible after collection. Dry weights were determined from parallel oven-dried samples (48 h at 60 °C).

Confirmation that the taxol fraction was retained throughout the extraction procedure was done by spiking samples with a known concentration of a taxol standard at different stages of sample preparation. In all cases 95+% recovery was obtained. Verification that taxol could be taken up by nodule tissue, and that nodule tissue could grow when taxol was present in the tissue, was done by spiking cultures with 20 μM taxol in 50 μL of DMSO, both at the start of induction from needles and in mature nodule cultures. In both cases, a significant taxol peak was recovered from the nodule tissue: 10% of the total spike in newly induced nodules (some into parent needles as well) and 90% of the total spike in mature nodules.

Taxol Induction Treatments. Starting parameters for all experiments were 5 g of nodule tissue in 50 mL of spent medium, 40 mL of sterile dH_2O, and 10 mL of refreshment medium, under incubation conditions previously described. Unless otherwise noted, 10 mL of spent medium was removed and replaced with 10 mL of refreshment medium every 2 weeks, and experiments were carried out for 12 weeks.

Several media modifications were tested to determine their effect on nodule growth and taxol production. Preliminary experiments compared refreshments with full-strength refreshment medium, hormone-free medium, and sterile distilled water, all supplemented with 2% sucrose. These experiments also investigated the role of medium refreshment in the production of taxol. Subsequent experiments (described here) compared refreshments of medium and distilled water, both with various sucrose levels (2%, 5%, and 10% sucrose), to give a final sucrose concentration in the culture of 0.2%, 0.5%, and 1.0% after refreshment.

To determine if taxol levels induced with the higher sucrose could be maintained or elevated, nodule cultures from medium with the normal complement of nutrients and hormones were given a refreshment treatment containing 2% or 5% sucrose (medium sucrose concentrations of 0.2% and 0.5% after refreshment). These cultures were maintained on the refreshment regime for 12 weeks with the two sucrose levels and sampled at 6, 10, and 12 weeks for taxol production. A subset of cultures were exposed to weekly refreshments and were refreshed with medium containing the hormones and nutrients at half the normal strength. Percent Brix, measured using a hand refractometer, was used as an indirect method of monitoring sucrose levels.

To determine if the observed effects on taxol production were due to a change in osmotic potential of the medium caused by the elevated sucrose levels, cultures were refreshed with medium osmotically adjusted with mannitol and sorbitol to equal the osmoticum of 0.5% and 1.0% sucrose. To make this adjustment, 44 mM each of mannitol and sorbitol, in addition to 58 mM sucrose, was added for the 0.5% sucrose equivalent osmotic medium, and 117 mM each mannitol and sorbitol plus 58 mM sucrose was added for the 1.0% sucrose equivalent medium. The 58 mM sucrose concentration was constant in the treatments and is equivalent to the 0.2% sucrose used under the control conditions. Subcultures were made of nodules originally refreshed with both 2% sucrose and 5% sucrose and were placed on refreshment regimes with either sucrose or the osmotic equivalent in mannitol, sorbitol, and sucrose. Cultures were sampled at 3 and 8 weeks.

In further experiments, nodule cultures were supplemented with several precursors and elicitors, including phenylalanine, potassium acetate, and methyl jasmonate, to test their effect on taxol production. Phenylalanine at 100 μM and 1 mM and potassium acetate at 10 and 100 mM were filter sterilized directly into the refreshment medium and added at each refreshment. Methyl jasmonate at 0.5 and 5.0 μM was added to medium at the time of initial subculture only. All cultures were maintained with regular refreshment regimes for a full 12 weeks except where culture senescence caused the early termination of some treatments.

Cultures of *Leptographium terebrantis* (provided by Dr. Ken Raffa, University of Wisconsin) were grown at 25 °C on potato dextrose agar or in potato dextrose broth.

All experiments were set up with at least three replicates. ANOVA analyses were done using Sigmastat (Jandel Scientific), and significance levels were determined at 0.05%.

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References and Notes

(1) We use the name taxol to refer to the chemical compound as named by Wani et al.[3] Taxol is a registered trademark of Bristol Myers Squibb Company.
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