Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*

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***Pestalotiopsis microspora* occurs as a range of strains in bald cypress, *Taxodium distichum*. The organisms live as endophytes in the bark, phloem and xylem, and isolates show differences in cultural and microscopic characteristics on common laboratory media. Many of these fungi make taxol as determined by the reactivity of partially purified culture extracts with specific monoclonal antibodies against taxol. In the case of one strain of *P. microspora* (CP-4), taxol was isolated from culture medium and was shown to be identical to authentic taxol by chromatographic and spectroscopic means.**

Keywords: taxol, *Pestalotiopsis microspora*, anticancer drugs, endophytic fungi, *Taxodium distichum*

INTRODUCTION

Taxodium distichum (L.) Rich (bald cypress) is a tall (30–40 m) coniferous tree typically growing in swamps, where it forms extensive pure stands. Its location is primarily in the SE United States (Harlow & Harrar, 1950). *Taxus* (yew), the source of the anticancer drug, taxol, belongs to the plant family Taxaceae, is also a conifer and grows in similar habitats to bald cypress (Harlow & Harrar, 1950; Hartzell, 1991). Both genera are evergreens with linear two-ranked leaves and both prefer damp environments. It is conceivable that the taxol in *Taxus* spp. may serve as a protective measure to the tree, given its effectiveness against the phycomycetous fungi *Pythium* and *Phytophthora*, which can cause pathological problems to roots of some plant species in damp environments (Young *et al.*, 1992). It is now known that several endophytes of *Taxus* spp. also produce taxol (Stierle *et al.*, 1993, 1994; Strobel *et al.*, 1996).

The acquisition of the metabolic machinery to produce taxol, either by coevolution or by genetic transfer, by these endophytes may enable them to better compete in their environment (Young *et al.*, 1992). Given the enormous reproductive potential of these fungi via spore formation, it is conceivable that taxol-producing endophytes might be found in plants not making taxol, but having a similar ecology to *Taxus*. Thus, our rationale was to search the swamps of the southern United States for such plants. The evergreens most resembling *Taxus*, but not producing taxol, are bald cypress – *T. distichum* and its relatives. Sampling a few limbs of this tree yielded

many strains of endophytic *Pestalotiopsis microspora*, some of which produced taxol. Thus, this report provides evidence of taxol production by fungi not associated with a plant that makes taxol, and indicates that other plant species residing in certain damp environments may also be hosts to such taxol-producing endophytes.

METHODS

Sampling of *T. distichum*. Samples of *T. distichum* were obtained several miles inland, in a swamp forest, from the central coast of South Carolina at the coordinates N 32° 52' 267" and W 80° 05' 334". We harvested seven terminal twig samples (0.5–1 cm × 20 cm) from a relatively young tree 7 m high. The samples were immediately transported to our laboratory in Montana and stored at 6 °C until being processed a few days later.

Endophytes of *T. distichum*. These fungi were isolated from the sterilized outer bark (Cb designation), the phloem/cambium (Cp designation) and the xylem (Cx designation). The stem fragments were surface-sterilized with 70 % (v/v) ethanol, then each layer of tissue was sampled with a sterilized sharp blade. Small pieces of plant tissue were excised and placed on water agar in Petri plates. After several days, individual hyphal tips of the fungal colonies were removed and placed on potato dextrose agar (PDA), incubated for 10 d, and periodically checked for culture purity. Eventually, each culture type was again transferred, by the hyphal tip method, to a water agar plate containing small pieces of sterilized carnation leaves. These gamma-irradiated leaves commonly permit the development of fungal fruiting structures which aid in their identification (Nelson *et al.*, 1983). The fungal isolates were numbered and stored in distilled H₂O at 4 °C as agar plugs or in 15 % (v/v) glycerol at –70 °C as spores and mycelium.

Fungal identification by microscopy. Fungal spores and fruiting bodies appearing on the carnation leaf fragments were examined by stereo and light microscopy for measurement and identification. Reference strains from the Centraalbureau voor Schimmecultures, Baarn, The Netherlands, were used for comparison. Comparisons of culture characteristics were made by standard methods after growing the organisms on PDA at 23 °C. Fruiting structures were fixed and processed using the methods of Upadhyay *et al.* (1991), except that they were placed in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2).

Fungal culturing and taxol isolation. The fungi were grown in 2-litre Erlenmeyer flasks containing 500 ml MID medium supplemented with 1 g soytone l⁻¹ (Strobel *et al.*, 1996). After 3 weeks of still culture at 23 °C, the culture fluid was passed through four layers of cheesecloth to remove solids and the preparation was treated as previously described (Strobel *et al.*, 1996). After methylene chloride extraction the extract of each fungal isolate was examined for the presence of taxol by an immunological method. In the case of one isolate, CP-4, we obtained at least 10 l of culture fluid and then isolated and purified the taxol present in the fluid by standard methods (Strobel *et al.*, 1996). This fungus was chosen for taxol isolation because of its relatively high production of taxol as determined immunologically on culture extracts.

TLC. All comparative TLC analyses were carried out on Merck 0.25 mm silica gel plates developed in the following solvents: A, chloroform/methanol (7:1, v/v); B, chloroform/acetonitrile (7:3, v/v); C, ethyl acetate/2-propanol (95:5, v/v); D, methylene chloride/tetrahydrofuran (6:2, v/v); and E, methylene chloride/methanol/dimethylformamide (90:9:1, by vol.). Taxol was detected using a spray reagent consisting of 1% vanillin (w/v) in sulfuric acid after gentle heating (Cardellina, 1991). It appears as a bluish spot fading to dark grey after 24 h.

HPLC. We used a taxil column (4.6 × 250 mm; Metachem Technologies) for determining the behaviour of putative fungal taxol by HPLC. Samples in 10 µl of methanol were injected and elution was done isocratically with methanol/H₂O (66:34, v/v). A variable-wavelength recorder set at 235 nm was used to detect compounds eluting from the column.

Spectroscopic analyses. After isolation from fungal cultures, the putative taxol was dissolved in 100% methanol and examined with a Beckman D-50 spectrophotometer. Electro-spray mass spectroscopy was done on samples dissolved in methanol/H₂O/acetic acid (50:50:1, by vol.). It was injected with a spray flow of 2 µl min⁻¹ with a spray voltage of 2.2 kV by the loop injection method.

Taxol immunoassays. A fungal extract was dissolved in methanol at 50 mg ml⁻¹. The insoluble materials were removed by centrifugation in a microfuge for 10 min. Taxol in samples was assayed by competitive inhibition enzyme immunoassay (CIEIA) method (Grothaus *et al.*, 1993). The assay was carried out using a taxol immunoassay kit (Hawaii Biotechnology Group) according to the procedure recommended by the suppliers. In brief, this assay was performed in a 96-well microtitre plate coated with taxol-protein coating antigen. The plate was blocked with 1% (w/v) BSA in PBS. After washing, the solid-phase-bound taxol was incubated with samples and taxol standards and a specific antitaxol monoclonal antibody. The taxol in the sample competes with solid-phase-bound taxol for binding to the monoclonal antibody. The monoclonal antibody bound to the solid-phase-bound taxol was detected by an alkaline-phosphatase-conjugated second antibody and alkaline phosphatase substrate, *p*-nitrophenol phosphate. The

inhibition of colour development was proportional to the concentration of free taxol present in samples. The amount of taxol in each sample was calculated from an inhibition curve made by using different concentrations of standard taxol supplied with the kit. We used this technique to screen for taxol in each of the fungal extracts. The assay is sensitive to about 1 ng ml⁻¹.

Materials. All solvents used for TLC and HPLC were HPLC-grade solvents; those used for extraction were ACS grade. Taxol used for reference purposes was a generous gift of the National Cancer Institute.

RESULTS AND DISCUSSION

Endophytic fungi

From seven limb pieces of bald cypress that were sampled, we recovered 21 endophytes, 16 of which were identified as *Pestalotiopsis microspora* (Speg.) Bat. & Peres. (Table 1). Typically, each individual isolate produced, on carnation leaves, conidiomata containing numerous conidia. The conidia were fusiform, possessing four septa with hyaline basal and terminal cells, with a brownish coloration in the median cells. The conidia had three or four characteristic terminal cell appendages (Strobel *et al.*, 1996). The conidia from each of the *P. microspora* strains generally averaged 6–7.6 µm × 18.7–28.8 µm in size when obtained from the carnation leaves (Table 1). Each of the isolates had spore sizes, shapes and coloration typical of *P. microspora* (Table 1).

Two of the other endophytes recovered from the limb pieces samples in this study were pycnidia formers making no taxol. They were studied no further.

Culturing of the *Pestalotiopsis* strains on a number of common laboratory media revealed that each had more or less distinctive cultural features. These were carefully noted using standard macromorphological characteristics after growth on PDA for 7 d at 23 °C. Of the strains of *Pestalotiopsis* shown in Table 1, with two exceptions, each was distinctive in some manner from the others listed. Only two isolates, Cb-8 and Cb-5, were identical in all respects. These characteristics were maintained after each of several transfers on PDA and other common laboratory media. However, with the other isolates listed, variation was noted in surface texture, colour (bottom of the culture), shape of the surface mycelia, zonation ridging or furrows, and sectoring (Table 1).

The behaviour of *P. microspora* (Cp-4) spores under different conditions of development, humidity and processing was also noted. Characteristically, when the conidia of *P. microspora* were formed they were turgid, but as cultures dried, conidia, both in leaf tissue and on the culture media, shrivelled. When placed in distilled water the conidia became turgid in a few seconds. However, in spore mounting media it took 1–2 h for spores to become turgid. Spores from dried cultures often burst after 10–15 min in distilled water. In light microscopic sections young developing conidia characteristically appeared turgid, and older spores, even on the same leaf tissue, were normally shrivelled. These same observations were

Table 1. Cultural characteristics of the *P. microspora* endophytes of *T. distichum*

Fungal strain*	Top surface texture	Bottom colour	Colony shape	Zonation†	Furrows†	Taxol (ng l ⁻¹)	Sectors†	Mean spore size (µm)‡
Cp-4	Tufted	Tan/brown sectors	Plateau	N	N	1487	Y	19.52 × 6.08
Cx-2	Tufted	Tan	Concave	Y, coarse	N	498	N	18.72 × 5.92
Cp-2	Tufted	Brown	Plateau	N	N	116	N	26.40 × 7.04
Cb-4	Tufted	Tan	Plateau	N	N	99	N	22.40 × 7.04
Cp-10	Tufted	Tan centre/ velvety margin	Concave	Y, coarse	Y	85	N	24.80 × 7.44
Cp-6	Tufted	Tan	Convex	N	N	82	N	26.24 × 6.40
Cb-2	Tufted	Brown centre/ tan margin	Concave	N	N	59	N	24.32 × 6.88
Cp-1	Tufted	Brown centre/ tan margin	Convex	N	N	24	N	24.16 × 7.20
Cx-1	Velvety centre/ tufted margin	Tan	Concave	Y, coarse	N	14	N	23.52 × 7.52
Cp-7	Tufted	Tan margin/ yellow centre	Concave	N	Y	0	N	26.56 × 6.80
Cp-5	Tufted	Tan margin/ brown centre	Concave	Y, coarse	N	0	N	24.16 × 6.72
Cb-3	Tufted	Tan margin/ yellow/white	Concave	Y, coarse	N	0	N	25.76 × 7.28
Cx-4	Tufted	Tan	Concave	N	N	0	Y	21.28 × 6.56
Cp-8	Velvety	Brown	Convex	N	N	0	N	28.80 × 6.48
Cb-8	Tufted	Tan uniform	Concave	N	N	0	N	24.32 × 6.80
Cb-5	Tufted	Tan uniform	Concave	N	N	0	N	24.16 × 6.72

* The isolation site of the strains is designated as follows: Cb, outer bark; Cp, phloem/cambium; Cx, xylem.

† N, no; Y, yes.

‡ Measurements were made on at least 10 spores per photograph. The spores were obtained from sterilized, inoculated carnation leaves. For comparative purposes, note that CBS isolate 790.68 of *P. microspora* has a mean spore size of 25.76 × 6.72 µm, and CBS isolate 332.76 has a size of 20.00 × 5.04 µm.

also made with tissues processed for SEM. However, newly developed spores remained turgid although the hyaline apical and basal cells sometimes shrivelled. Although some studies have been done on the ultra-structural characteristics of *Pestalotiopsis* (Sun *et al.*, 1990; Purohit & Chawla, 1989), none seem to have observed this behaviour of conidiospore shrinkage and swelling.

Taxol from endophytes

An enormous variation was noted in taxol production by the *Pestalotiopsis* strains as measured by the immunological assay (Table 1). This variation in taxol production of *Pestalotiopsis* will offer an important tool in unravelling the molecular biology of taxol producing fungi via mRNA differential display techniques (Liang *et al.*, 1993).

As a further check on validity of the immunoassay, we chose to isolate and characterize taxol from the highly productive taxol strain Cp-4 (Table 1). We prepared 10 l of 3-week-old culture fluid and processed it as described by Strobel *et al.* (1996). The compound that was isolated, in microgram quantities, possessed identical chroma-

tographic properties in all TLC systems (A-E), and yielded an identical colour reaction, to authentic taxol. It was never seen in uninoculated culture media and could not have been carried from the original source tree, since taxol is not present in bald cypress. This fact was established after we exhaustively extracted 5 g of cut leaves and twigs of bald cypress with methylene chloride and subjected the residue to the immunological analyses, which gave negative results.

The fungal compound produced an identical electrospray mass spectrum as authentic taxol, with the major molecular ion being $(M+Na)^+ = 876$ (McClure *et al.*, 1992; Stierle *et al.*, 1993). The $(M+H)^+$ peak was not observed either with authentic taxol or with the fungal preparation. Furthermore, the compound from the fungus yielded a UV absorption spectrum identical to that of authentic taxol (Wani *et al.*, 1971). We also observed that the fungal compound gave a single peak when eluting from the taxsil HPLC column, with about the same retention time as authentic taxol. When authentic taxol, in equimolar amounts, was co-chromatographed with the fungal compound only a single peak appeared, having a retention of

25.8 min. The combined immunochemical, chromatographic and spectroscopic data suggest that the fungal compound is taxol.

Conclusions

The observations presented in this report strongly suggest that plants other than yew may be a source of microbes producing taxol. An examination of Western red cedar, *Thuja plicata*, a common species in the canopies of Western yew forests, for *P. microspora* and other endophytes has yielded only a few microbes and no *Pestalotiopsis* spp. (G. Strobel, unpublished). Seemingly the cedars are not a reservoir for the microbes of yew even though their large size and predominance in the locales common to the yews would expose them to the same spectrum of fungi.

There is no doubt that *P. microspora* strain Cp-4 makes taxol. However, this strain also is one that frequently makes sectors on PDA plates, indicating that it is genetically unstable. This fact may account for the wide range of taxol production observed from this isolate, varying from 50 to 1487 ng l⁻¹ (J. Li & R. Sidhu, unpublished). This sectoring phenomenon seems to be relatively uncommon among the other isolates of *P. microspora* (Table 1).

We also speculate that *P. microspora* and other species of the *Pestalotiopsis*/*Pestalotia* complex may be an extremely genetically flexible group, as manifested by their large variation in cultural characteristics and taxol production (Table 1). This flexibility might be related to their peculiar cell morphology and their ability to respond dramatically to environmental conditions. Such responses may represent a ready exchange of genetic information allowing this organism to mimic the chemical environment of its host and, ultimately to gain a number of evolutionary advantages.

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REFERENCES

- Cardellina, J. H. (1991). HPLC separation of taxol and cephalomannine. *J Liq Chromatogr* **14**, 659–665.
- Grothaus, P. G., Raybould, T. J. G., Bignami, G. S., Lazo, C. B. & Byrnes, J. B. (1993). An enzyme immunoassay for the determination of taxol and taxanes in *Taxus* sp. tissues and human plasma. *J Immunol Methods* **158**, 5–15.
- Harlow, W. M. & Harrar, E. S. (1950). *Textbook of Dendrology*. New York: McGraw-Hill.
- Hartzell, H. (1991). *The Yew Tree*. Eugene, OR: Hugolosi Press.
- Liang, P., Avenbourn, L. & Pardee, A. B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Res* **21**, 3269–3275.
- McClure, T. D., Schram, K. N. & Reimer, M. L. J. (1992). The mass spectroscopy of taxol. *Am Soc Mass Spectrom* **3**, 672–679.
- Nag, T. R. (1993). *Coelomycetous Anamorphs with Appendage Bearing Conidia*. Waterloo, Ontario: Mycologue Publications.
- Nelson, T. A., Toussoun, T. & Marasas, W. F. O. (1983). *Fusarium Species*. University Park, PA: Penn State University Press.
- Purohit, D. K. & Chawla, G. C. (1989). Fine structure of conidia in two pathogenic species of *Pestalotiopsis*. *Curr Sci* **58**, 659–665.
- Stierle, A., Strobel, G. & Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* **260**, 214–216.
- Stierle, A., Stierle, D., Strobel, G., Bignami, G. & Gothaus, P. (1994). Bioactive metabolites of the endophytic fungi of Pacific yew, *Taxus brevifolia*. In *Taxane Anticancer Agents, Basic Science Current Status* (American Chemical Society Symposium Series, no. 583), pp. 81–97. Edited by G. I. Georg, T. T. Chen, I. Ojima & D. M. Vyas. Washington, DC: American Chemical Society.
- Strobel, G. A., Yang, X., Sears, J., Kramer, R., Sidhu, R. & Hess, W. M. (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology* **142**, 435–440.
- Sun, X. A., Ge, Q. X. & Ling, H. Z. (1990). Studies on the criteria of morphological characteristics for species classification of *Pestalotiopsis*. I. A brief review of nomenclature for the genus *Pestalotiopsis* and observations on conidia of *Pestalotiopsis* by SEM. *Acta Agric Univ Zhejiangensis* **16** (Suppl. 2), 168–172.
- Upadhyay, R. K., Strobel, G. A. & Hess, W. M. (1991). Morphogenesis and ultrastructure of the conidiomata of *Ascochyta cypericola*. *Mycol Res* **95**, 785–791.
- Wani, M. C., Taylor, H. L., Wall, M. E., Coggen, P. & McPhail, A. T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* **93**, 2325–2327.
- Young, D. H., Michelotti, E. J., Swindell, C. S. & Krauss, N. E. (1992). Antifungal properties of taxol and various analogues. *Experientia* **48**, 882–885.

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