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The induction of taxol production in the endophytic fungus—*Periconia* sp from *Torreya grandifolia*

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A *Periconia* sp was isolated from *Torreya grandifolia* (a relative of yew that does not synthesize taxol) near Huangshan National Park in the People's Republic of China. This fungus, not previously known as a tree endophyte, was isolated from the inner bark of a small lower limb. When freshly isolated from the tree and placed in a semi-synthetic medium, the fungus produced readily detectable quantities of the anticancer drug taxol. Other taxol-producing endophytes were also isolated from this source. The production of taxol by *Periconia* sp was demonstrated unequivocally via spectroscopic and immunological methods. However, successive transfers of the fungus in semi-synthetic medium resulted in gradual attenuation until low production occurred even though fungal growth was relatively unaffected. Several compounds, known previously as activators of microbial metabolism, including serinol, *p*-hydroxybenzoic acid, and a mixture of phenolic acids, were capable of fully or partially restoring taxol production to otherwise taxol-attenuated cultures. The compound with the most impressive ability to activate taxol production was benzoic acid at 0.01 mM. Benzoic acid was not a taxol precursor.

Keywords: taxol; attenuation; benzoic acid; *Periconia* sp; endophyte

Introduction

Taxol is a complex diterpenoid [14] produced by all species of *Taxus*. This compound is a potent antifungal agent and possibly serves the tree by protecting it against the effects of potential pathogens, especially the water molds (oomycetes) [15]. The mode of action of taxol on the oomycetes appears to be identical to its activity against a number of human cancer cells by preventing the depolymerization of tubulin during cell division [9]. Although taxol has been totally synthesized [7], all of it currently on the market has its origins, completely or in part, from *Taxus* spp.

The amount of taxol found in yews is relatively small, *ca* 0.01–0.03% dry weight and this has been a major factor contributing to its high price. With the discovery that certain endophytic fungi are able to produce taxol has come the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation [10,12]. To date, all taxol production by fungi, representing such genera as *Taxomyces*, *Pestalotiopsis*, *Monochaetia* and *Alternaria*, is constitutive under the standard conditions of fermentation that have been used [11]. However, the subject of this report, not only unequivocally demonstrates taxol production by a novel endophytic fungus of trees, *Periconia* sp, but also shows that taxol production seems to be inducible in this fungus.

Materials and methods

The fungus used in this work was one of many recovered from a 1.0-cm (dia) × 30.0-cm (length) limb removed from a 15–20 m high *Torreya grandifolia* tree growing between Huangshan City and Huangshan Park in SE mainland China (PRC). The stem was surface-treated with 70% ethanol (v/v), the outer bark was removed with a sterilized sharp blade, and the inner bark/phloem-cambium tissue pieces were placed on water agar in Petri plates. After several days incubation at 23°C, individual hyphal tips of the developing fungal colonies were removed and placed on potato dextrose agar (PDA), incubated for 8–10 days, and periodically checked for culture purity. Eventually, each pure culture was transferred again, by hyphal tipping, to a Petri plate containing water agar and small pieces of sterilized carnation leaves. The gamma-irradiated leaves commonly encourage the development of fungal fruiting structures which aid in their identification [12]. The fungal isolates, obtained from *T. grandifolia*, were numbered and stored in distilled water at 4°C as agar plugs and 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 Schimmelcultures (CBS), Baarn, The Netherlands, were used for comparison. Comparisons of the 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* [13], except that they were placed in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The samples were critical point dried, gold coated with a sputter coater and observed and photographed with a JEOL 6100 scanning electron microscope.

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Fungal culturing and taxol isolation

The fungi were grown in 2-L Erlenmeyer flasks, as previously described, containing 500 ml MID medium which included 1 g soytone L^{-1} [12]. After 3 weeks of still culture at 23°C, the culture fluid was passed through four layers of cheesecloth to remove solids. After methylene chloride extraction the culture fluid of each fungal isolate was examined for the presence of taxol by an immunological method [11].

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, ethylacetate/2-propanol (95:5, v/v); D, methylene chloride/tetrahydrofuran (6:2, v/v); and E, methylene chloride/methanol/ dimethylformamide (90:9:1, v/v/v). Taxol was detected using a spray reagent consisting of 1% vanillin (w/v) in sulfuric acid after gentle heating [2]. It appeared as a bluish spot fading to dark grey after 24 h.

Spectroscopic analyses

After isolation from fungal cultures, the putative taxol was dissolved in 100% methanol and examined with a Beckman D-50 spectrophotometer. Electrospray mass spectroscopy was carried out on samples dissolved in methanol/ H_2O/acetic acid (50:50:1, v/v/v). Samples were injected with a spray flow of 2 μl min^{-1} and a spray voltage of 2.2 kV by the loop injection method. Nuclear magnetic resonance spectroscopy (NMR) was done on taxol preparations in a Brucker 500 MHz instrument with the sample dissolved in 100% deuterated methanol. The sample was subjected to 2048 scans with a sweep width of 6024 and 8 K real points.

Taxol immunoassays and quantitation

Crude fungal extracts were 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 a competitive inhibition enzyme immunoassay (CIEIA) method [6]. 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. Taxol in the sample competed with solid-phase-bound taxol and was detected by an alkaline phosphatase-conjugated second antibody and alkaline phosphatase substrate, ρ-nitrophenol phosphate. The inhibition of color 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}.

Plant extracts

An aqueous extract of *Torreya taxifolia* was made by soaking 20 g of chopped leaf and stem fragments in 1 L of water for 3 days at 20°C. The sample was filtered through four layers of cheesecloth and dried by flash evaporation at 40°C. Each sample was taken up in a small volume of water and then lyophilized to produce a whitish powder. Some samples were further separated into cationic, anionic, neutral and lipid fractions. Initially, the aqueous extract was treated with three equal volumes of CH_2Cl_2 and this solvent, after separation, was taken to dryness by flash evaporation and considered as the 'lipid fraction'. The remaining aqueous sample was separated by sequential passages over 3 cm × 6 cm columns of Dowex 50 H, and Dowex 1-formate. Elution of the Dowex 50 was done with 6 N TFA yielding the 'cationic fraction', while Dowex 1 was eluted with 6 N formic acid yielding the 'anionic fraction'. The 'neutral fraction' freely passed through both columns.

Radiolabeling experiments

Various ^{14}C-labeled compounds were tested, on a comparative basis with ^{14}C-benzoate, as precursors to fungal taxol. Each of the ^{14}C-compounds was added to the culture medium just prior to inoculation with *Periconia* sp. The labeled compounds (20 μCi) were dissolved in 90% ethanol (which sterilized them) and added in a volume not exceeding 0.1 ml to each of two 2-L flasks containing 500 ml of culture medium. The culture medium was harvested for taxol according to the methods described above. The ratio of the specific radioactivity of the fungal taxol (product) to the specific radioactivity of the potential precursor was determined. In this manner, an evaluation of the most and least likely potential precursors could be ascertained, especially ^{14}C-benzoic acid.

All radioactivity measurements were made with compounds dissolved in 1.0 ml of absolute methanol and placed in 10 ml of aquasol (New England Nuclear, Boston, MA, USA). Each sample was counted for 100 min on a Packard Tri-Carb liquid scintillation counter and all counts corrected to dpm by the quench correction method. Taxol, in these experiments, was quantified by its millimolar extinction coefficient at 273 nm.

Statistical methods

Each experimental data point is the result of an average of three replicated flasks done simultaneously. The standard deviation of the mean is shown in the table and as error bars in the figures.

Materials

All solvents used for TLC were HPLC grade. Those used for extraction were ACS grade. Taxol for reference purposes was a generous gift from the National Cancer Institute.

Results

Torreya and *Taxus* are the only two genera in the family *Taxaceae*. Although all *Taxus* spp produce taxol, little or no taxol is detectable in representative species of *Torreya*. *Torreya* and *Taxus* generally share the same type of habi-

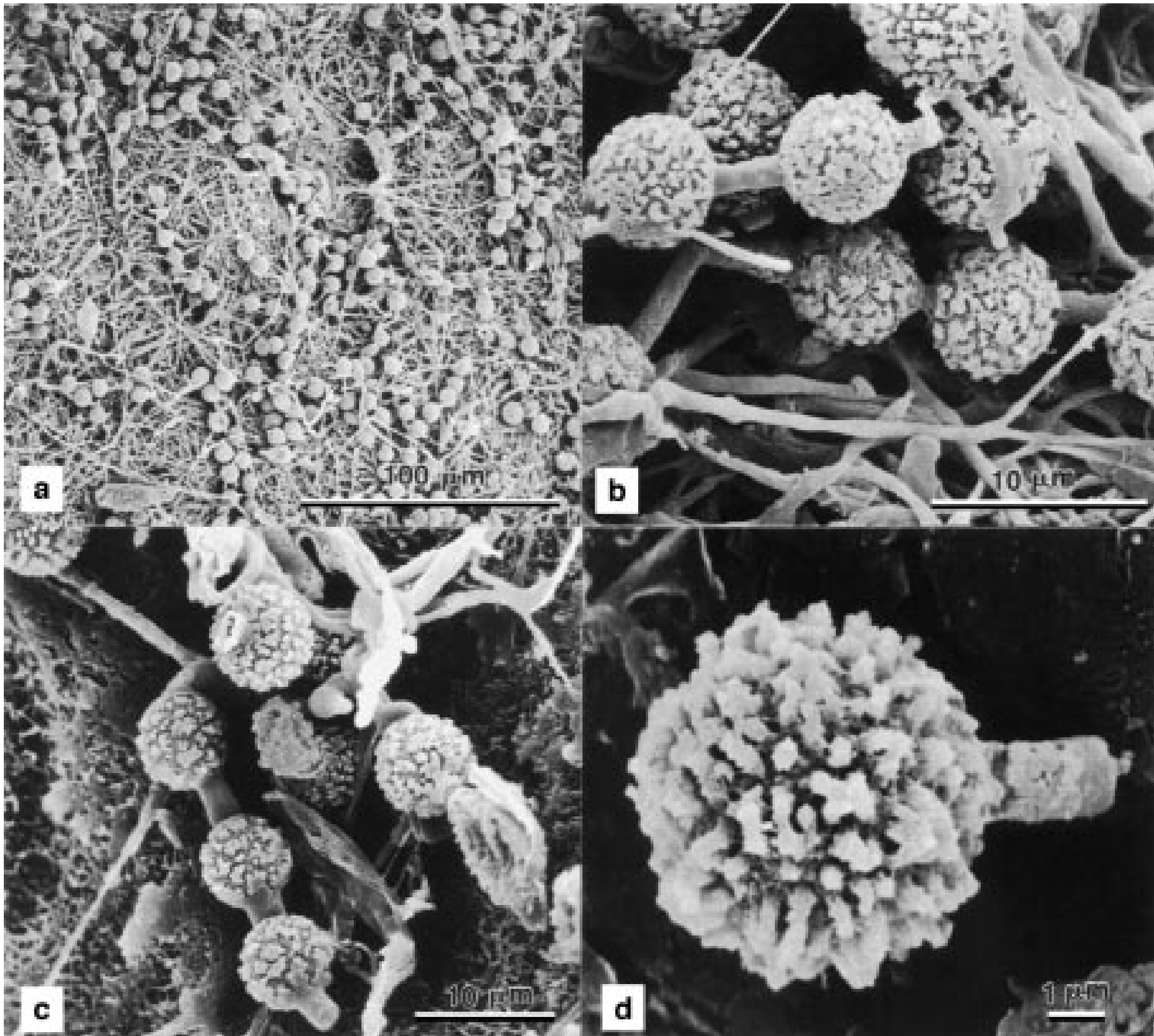

Figure 1 Scanning electron micrographs of *Periconia* sp. (a) Conidiophores developing in long chains on PDA medium. (b) An enlarged view of conidiospore attachment on PDA medium. (c) Conidiospore production on carnation leaves resulting in either individual spores or ones in short chains. (d) An individual conidiospore. Magnifications are shown by the bars on each illustration.

tats, moist hillsides or valleys at 1000–3000 m elevation. In mainland China, several species of *Torreya* occur and they are so common that they are harvested for timber and fuel. Since several taxol-producing fungi have been found as endophytes of *Taxus* sp [11], it was of interest to learn if *Torreya* sp also supported taxol-producing endophytic fungi.

From one limb piece of *T. grandifolia* that was sampled, 25 cultures of endophytic fungi were recovered. The most frequently recovered endophytic genus was *Pestalotiopsis* spp (7/25) with one of these being a taxol producer. Others found were *Phoma* sp, *Fusarium* sp, *Acremonium* sp, *Nigrospora* sp, and several sterile fungi (producing no fruiting structures), none of which produced taxol. However, at least four other sterile fungi were modest taxol producers. Of the fungi isolated, the most interesting was *Periconia* sp since it is not commonly known as an endophytic fungus associated with tree species. Its identity was confirmed by Dr W Gams of the CBS, Baarn, The Netherlands. On PDA it characteristically produced conidiospores in long chains (Figures 1a and b). However, on the carnation leaves in water agar, individual conidiospores were dark, long, stout, and shortly-branched near the apex, bearing one or more loosely connected groups of conidia (Figure 1c). The individual conidia were highly decorated having protrusions and commonly bearing a tubular segment of the conidiophore if they were borne on PDA (Figure 1d). This isolated *Periconia* sp was deposited in the Montana State University mycological collection as No. 2026.

Taxol from Periconia sp

A compound having chromatographic properties identical to authentic taxol in solvent systems A–E, and giving a blue-grey color reaction with the vanillin/sulfuric acid reagent was consistently isolated from *Periconia* sp. Sterile culture media did not have such a compound. The purified compound isolated from this fungus yielded both UV and NMR spectra that were identical to authentic taxol [5,10].

Further convincing evidence for the identity of the compound as taxol was obtained by electrospray mass spectroscopy. Characteristically, authentic taxol yields both an $(M+H)^+$ peak at 854 and $(M+Na)^+$ peak at 876. The product of *Periconia* sp produced a spectrum identical to authentic taxol (Figure 2).

Periconia sp freshly isolated from its tree host, typically produced 300–400 ng taxol L^{-1} as estimated by the immunoassay procedure. This is 6–8 times greater than the yields of taxol originally observed being produced by *Taxomyces andreanae* [10] and about 100 times less than *Pestalotiopsis microspora* [12].

Attenuation of taxol production

When the original culture (isolated directly from *T. grandifolia*) of *Periconia* sp was grown for 3 weeks on MID medium, *ca* 350 ng taxol L^{-1} was produced (Figure 3). However, after successive serial transfers on this medium (after 1 week's growth) and then incubation in MID medium there was a visible reduction in the coloration of the medium which changed from reddish brown in the original culture to having virtually no pigmentation after three transfers. Furthermore, taxol production also steadily declined until only *ca* 118 ng taxol L^{-1} was produced after five transfers (Figure 3).

When the attenuated fungus, producing *ca* 118 ng taxol L^{-1} (Figure 3), was transferred back to MID medium containing instant potato extract, or PDB (potato dextrose broth) medium, taxol production fell to less than half that of MID medium alone (Table 1). However, when the attenuated fungus was placed in the presence of 100 mg L^{-1} of *Torreya taxifolia* (Florida *Torreya*) extract in MID medium, taxol production nearly tripled to *ca* 350 ng L^{-1}. Various fractions of the *T. taxifolia* extract were not as effective as the crude material in enhancing taxol production by *Periconia* sp, but the neutral fraction at 100 mg L^{-1} was the most effective of these fractions (Table 1). This result suggested to us that either the *Torreya* extract possessed a critical precursor to fungal taxol production, or that some activating substance(s) was present in it.

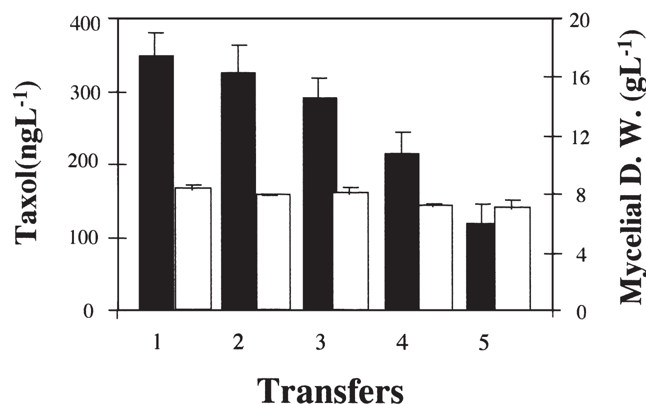

Figure 3 Serial transfers of *Periconia* sp from PDA plates grown for 1 week and then transferred to MID medium. The inoculated flask was then incubated for 3 weeks, and the taxol extracted and assayed (■). This was done starting from the fungus originally isolated from the *T. grandifolia* tree and subsequently transferred four times with 1-week intervals. The mycelial dry weight was also obtained (❐).

Activators (inducers) of taxol production

It has long been known that certain specific compounds in higher plants can influence the production of secondary metabolites by fungi. To this end, we tested several known activators of fungal metabolism for their effects on taxol production by an attenuated culture of *Periconia* sp. The most effective of these compounds was benzoic acid, at 0.01 mM, which caused an 8-fold increase in taxol pro-

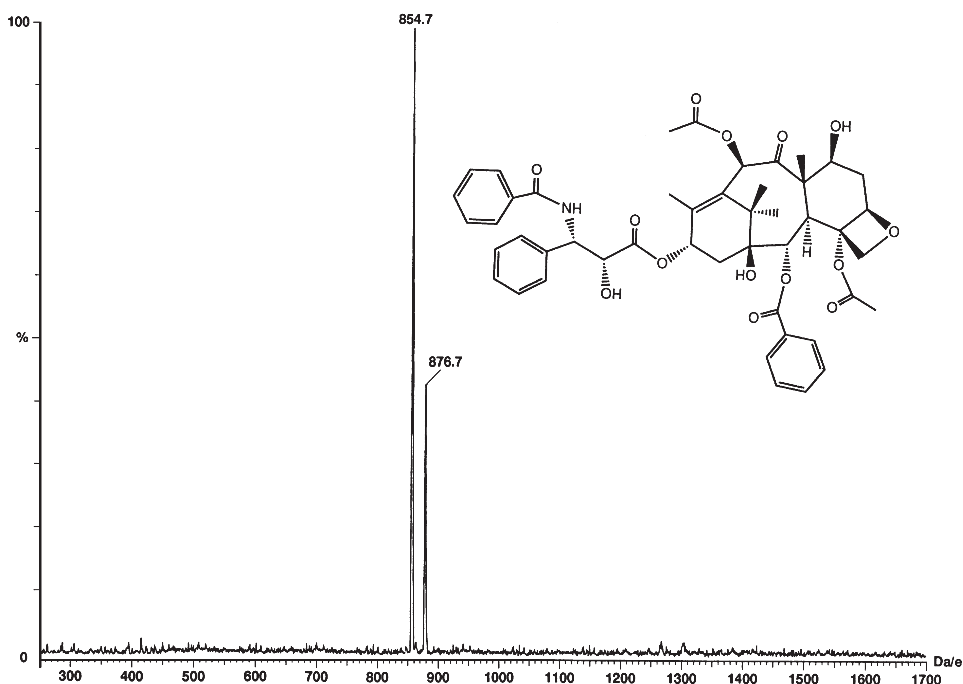

Figure 2 An electrospray mass spectrum of taxol isolated from *Periconia* sp. The structure of taxol is shown as an insert.

Table 1 The effects of various products and compounds on taxol production by an attenuated culture of *Periconia* sp[a]

Medium or compound added	Concentration	Taxol produced (ng L^{-1})
MID alone		118 ± 28
PDB potato dextrose broth	24 g L^{-1}	54 ± 12
MID plus instant potato extract	10 g L^{-1}	30 ± 21
Torreya extract	100 mg L^{-1}	347 ± 32
Organic acids (*Torreya* extract)	100 mg L^{-1}	76 ± 6.8
Amino acids (*Torreya* extract)	100 mg L^{-1}	97 ± 8.0
Lipids (*Torreya* extract)	100 mg L^{-1}	86 ± 7.7
Sugars (neutral *Torreya* extract)	100 mg L^{-1}	155 ± 14
Serinol	0.1 mM	256 ± 5
p-Hydroxy benzoic acid	0.20 mM	595 ± 82
β-resorcyclic acid	0.02 mM	291 ± 16
Gallic acid	0.1 mM	297 ± 38
Benzoic acid	0.01 mM	831 ± 27
Mixture of activators[b]	0.02 mM	521 ± 6

[a]In each case the contents of the 3-week-old culture medium were extracted with CH_2Cl_2 and tested for taxol via the immunological technique. Also, in the case of each *Torreya* fraction and all other individual compounds tested, they were placed at the concentrations given in the MID medium and inoculated with the fungus.
[b]The mixture of activators was collectively tested at 0.02 M and consisted of serinol, *p*-hydoxybenzoic acid, protocatechuic acid, catechol, *β*-resorcyclic acid and gallic acid.

duction (Table 1). This compound was followed by *ρ*-hydroxybenzoic acid, at 0.2 mM, a mixture of activators, and other compounds at the optimum concentrations at which they were effective (Table 1). However, a detailed examination of both *ρ*-hydroxybenzoic acid and benzoic acid over a range of four orders of magnitude concentration indicated that activation occurs in a narrow range of concentrations at 0.2 mM and 0.01 mM, respectively (Figures 4 and 5). Furthermore, at a concentration of 10 mM benzoic acid, taxol production fell to zero as all fungal growth ceased.

Taxol precursors in Periconia sp

In order to determine if benzoic acid administered to *Periconia* sp was truly acting as a metabolic regulator 'activator' (Table 1), and not simply as a precursor, ^{14}C-labeling experiments were conducted. Fungal cultures, producing optimal amounts of taxol, were administered a total of 20 μCi of either [7-^{14}C] Na benzoate, [U-^{14}C] glucose, or [U-^{14}C] phenylalanine with specific radioactivities of 22, 251, and 450 mCi mMol^{-1}, respectively, according to methods described above. The specific radioactivity of the taxol isolated from each of the culture broths was determined. Fungal growth was comparable in each of the flasks after 21 days. No ^{14}C-taxol was recovered from the flasks administered ^{14}C-Na benzoate. However, both ^{14}C-glucose and ^{14}C-phenylalanine served as precursors to ^{14}C-taxol in *Periconia* sp. Based on a comparison of the ratio of the specific radioactivity of the compound administered to the specific activity of the taxol recovered, ^{14}C-phenylalanine served as a better precursor than ^{14}C-glucose in taxol biosynthesis. This was also previously observed with *T. andreanae* as well as *P. microspora* [10,12].

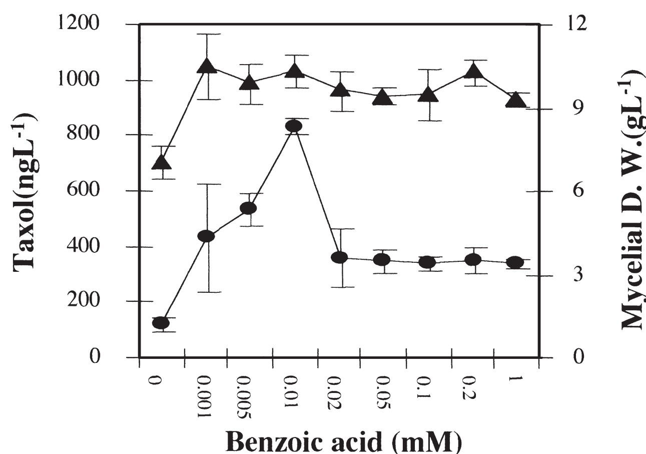

Figure 4 The induction of taxol production in MID medium plus various concentrations of benzoic acid in an attenuated culture of *Periconia* sp. Each flask was incubated for 3 weeks and then assayed for taxol (●—●). The dry weight was also obtained in each culture (▲—▲).

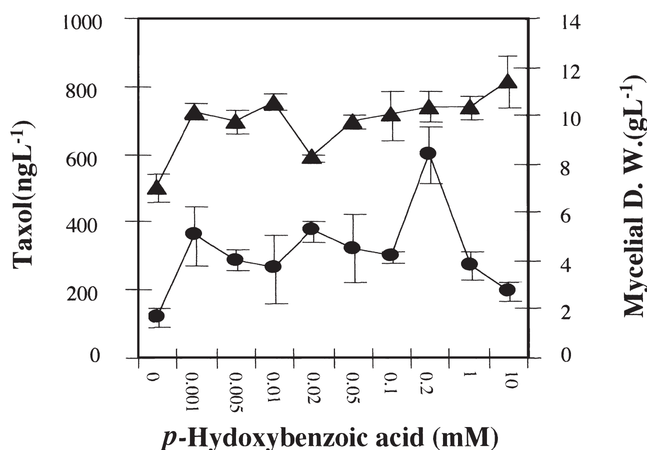

Figure 5 The same conditions as in Figure 4 were used in this experiment except that *p*-hydroxybenzoic acid was substituted for benzoic acid.

Discussion

The endophytic fungi of the majority of woody plant species in the world are yet to be studied. *Periconia* sp, the subject of this report, is most commonly associated with grasses, sedges and rushes and is unknown as an endophyte of any tree [5]. Yet, it was found within the phloem/cambium of *Torreya grandifolia*.

The ability of *Periconia* sp to make taxol was substantiated and confirmed by a number of spectroscopic tests including NMR, UV and mass spectroscopy. The most common endophytic fungus making taxol is *Pestalotiopsis microspora* and related species of *Pestalotiopsis* [11]. It remains unclear why, from an evolutionary genetics perspective, *Taxus* spp and selected endophytes of these plants share the ability to synthesize taxol. Two possible explanations for this are that taxol biosynthesis was horizontally transferred, or that each eukaryote independently evolved this trait. In principle, the two possibilities are distinguishable at the molecular (sequence) level, and the present work should help facilitate reaching this goal. *Periconia* sp is unrelated to *P. microspora* except that both are deuteromycetes. Uncertainty still prevails for the explanations as to

why and how taxol production in endophytic fungi has developed. One possibility is that horizontal gene transfer between *Taxus* spp and fungal endophytes has occurred rendering a certain population of fungi capable of producing taxol. Likewise, it is not inconceivable that a genetic transfer event can occur from a fungus to the plant. The former seems more likely since a widely diverse group of fungi are capable of taxol production and only *Taxus* sp (to date) are known taxol producers.

Although several plant-associated fungi use plant secondary products as activators or inducers of phytotoxin production [8], and several cases are known for the induction of important secondary metabolites in actinomycetes and other filamentous fungi [3,4], this is the first report of an endophyte utilizing one or more compounds as activators of taxol production. Several compounds, including serinol are known as activators of helminthosporoside production in *H. sacchari* and serinol has the same activation effect in *Periconia* sp (Table 1) [8]. However, the most effective activator, in our study, was benzoic acid. Originally, it was not clear whether benzoic acid was an activator or was being used by the fungus in a precursor–product relationship. Since taxol does have a benzoate moiety on it (Figure 2), it seemed reasonable to suggest it as a precursor. However, neither in *Taxomyces andreanae*, nor in *Periconia* sp could benzoate be shown to be a precursor, at least as much so as ^{14}C-glucose or ^{14}C-phenylalanine [10,12]. Phenolic compounds of the type tested (Table 1) induce virulence in *Agrobacterium tumefaciens* [1]. From a practical point of view, these results strongly suggest that taxol production in some fungi is regulated by plant products and may contribute towards the goal of realizing commercial taxol production via industrial fermentation.

Acknowledgements

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