Sensitivity of Plant Pathogenic Fungi to Taxane Extracts from Ornamental Yews

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ABSTRACT


Taxanes were extracted from the needles of ornamental yews using methanol followed by solid phase extraction (SPE) cleanup. The concentrations of paclitaxel (Taxol) and other taxanes, cephalomannine and baccatin III, were present typically in a 10:5:1 ratio along with several other taxane compounds. A 95% ethanol preparation of the extracts was amended into potato-dextrose agar (PDA), which was then characterized by its paclitaxel content. The radial growth of 12 plant pathogens in the Ascomycetes, Deuteromycetes, and Oomycetes were recorded and used to determine their EC50 values on the taxane-amended PDA. The fungi in the Ascomycetes and Deuteromycetes were classified as taxane insensitive, and their EC50 values were not determined within the concentration range examined (paclitaxel at EC50 > 4.00 μg/ml). The five Oomycetes examined were classified as taxane sensitive. *Pythium aphanidermatum* had the lowest EC50 (paclitaxel at 0.05 μg/ml [0.058 μM]), and *Pythium irregulare* had the highest EC50 (paclitaxel at 1.3 μg/ml [1.52 μM]). Compared with a pure standard of paclitaxel or a combination of the three authentic taxane standards, the partially purified taxane extract was more toxic to *P. aphanidermatum* and *Pythium irregulare*, but less toxic to *P. irregulare*. Authentic cephalomannine was less toxic than paclitaxel, and baccatin III had no significant effect on these *Pythium* spp. at concentrations up to 2.0 μg/ml. The wide variation of fungal sensitivity to the taxane extract suggests that different mechanisms and/or different target sites may exist across fungal species. Taxanes may offer a new chemistry for inhibiting the Oomycetes pathogens.

Additional keywords: Taxus spp.

Paclitaxel (Taxol), cephalomannine, and baccatin III (Fig. 1), and other related taxanes, are diterpenoids that can be extracted from the tissue of *Taxus* spp. Among these taxane compounds, paclitaxel has received considerable attention due to its unique mode of action as a mitotic spindle poison. Critical to the formation of the mitotic spindle is the tubulin<=>microtubule equilibrium. In contrast to other mitotic disrupters, such as the vinca alkaloids, which limit cell division by shifting the equilibrium toward tubulin, paclitaxel and taxanes shift the equilibrium toward the microtubule (18,20). The microtubules are stabilized against depolymerization back to tubulin and thus disrupt cytokinesis.

Paclitaxel is currently extracted from the bark of the Pacific yew tree (*Taxus brevifolia* Nutt.) for use in the treatment of ovarian cancer (4). Although the main interest in paclitaxel is as a stabilizer of microtubules in mammalian cells, preliminary studies showed that paclitaxel has cytostatic effects in a range of eukaryotic cells. The effects of paclitaxel on unfertilized and fertilized eggs of *Xenopus laevis*, the African water frog, (10) and of the sea urchin *Lytechinus variegatus* and *Arbacia punctulata* (17) have been reported. Paclitaxel was shown to block the replication of the human pathogenic hemoflagellate *Trypanosoma cruzi* (2). In plants, paclitaxel reduced the rate of mitosis in the endosperm of blood lilies, *Haemanthus* spp., (1) and stabilized the formation of microtubules in the Chrysophyllum aglae, *Poterioochromonas* spp. (11). It was demonstrated in the *Myxomycete* slime mold *Physarum polycephalum* that paclitaxel can stabilize the monomers and block the disassembly of amoebal microtubules (13,22). In the fungus *Uromyces phaeoscleri* (12), paclitaxel enhanced the microtubule array.

The fungitoxicity of taxane analogs was explored by Latas et al (13) and Young et al (23). Latas et al (13) reported that baccatin III, an analog of paclitaxel that lacked the biologically active side chain at carbon 13 of the taxane A ring (Fig. 1), had no activity against mammalian cells; but it was equal to paclitaxel in its ability to prevent the disassembly of microtubules from the slime mold *Physarum polycephalum*. Using authentic taxane analogs, Young et al (23) found wide variation among different fungi to several of the analogs. For example, *Aphanomyces cochlioides* and *Physomythora capitata* exhibited different sensitivities to certain analogs of baccatin III. Young et al (23) also reported that species in the Oomycetes were more sensitive to paclitaxel than certain species in the Ascomycetes, Basidiomycetes, or Deuteromycetes. Wagner and Flores (21) reported that the Oomycetes were more sensitive to standards of paclitaxel and cephalomannine, but they found that baccatin III was ineffective in suppressing growth.

MATERIALS AND METHODS

Extraction of taxanes. The details of extraction and cleanup of the *Taxus* biomass have been reported elsewhere (14). Briefly, needles of ornamental yew cultivars, such as *Taxus × media* 'Hicksii' and 'Nigra' from commercial plantings in Connecticut

Vol. 84, No. 10, 1994 1179
and Rhode Island, from field research plots at the Connecticut Agricultural Experiment Station's Lockwood Farm, and from greenhouse-rooted cuttings were weighed and blended to 3 mm or smaller in size. A sample of blended needles was extracted with methanol by agitation on a wrist-action shaker for 16 h at room temperature. The resulting solution was filtered through Whatman No. 1 filter paper, reduced to a solid residue by rotary evaporation at 43-45 C, and reconstituted in 80% methanol-deionized water. The reconstituted crude extract was fractionated by solid phase extraction (SPE) cartridges and the following eluants in turn: deionized water, 20% methanol, 50% methanol, and 80% methanol. The 80% methanol eluate, hereafter referred to as the taxane fraction, contained the taxanes and was collected, dried under rotary evaporation, and reconstituted in 95% ethanol for in vitro assays. The reconstituted taxane fraction was quantified for its paclitaxel and cephalomannine by high-pressure liquid chromatography (HPLC) using a Perkin-Elmer LC250 (Perkin-Elmer Corp., Norwalk, CT) with an LC235 photodiode array dual channel detector. For quantitation, the λ = 230 nm channel was used; the λ = 280 nm channel was also recorded. Gradient elution on a MetaChem Taxsl

![Baccatin III]

Fig. 1. Structures of paclitaxel, cephalomannine, and baccatin III.

(MetaChem Technologies, Inc., Torrance, CA) guard column and a MetaChem Taxsl LC column (250 × 4.6 mm, 5 μm) permits paclitaxel to be separated from co-eluting cinnamyl analogs for accurate quantification. Using an alternate HPLC program, we have demonstrated that the quantity of 7-epi-10-deacetyltaxol in *Taxus* needles is very low and will not affect paclitaxel quantitation significantly (M. J. I. Mattina, unpublished).

**Preparation for in vitro assay.** In vitro fungal inhibition studies were conducted by measuring radial growth across taxane-amended agar. For the study of *Taxus* extracts, dilutions of the reconstituted 95% ethanol taxane fraction were prepared in 95% ethanol so that the final volume equaled 1.3 ml. This was amended into 100 ml of cooled (48-50 C) molten potato-dextrose agar (PDA) contained in a 250-ml Erlenmeyer flask. After agitating the flask on a stir-plate for 10-15 s, 5-ml portions of the medium were transferred to plastic petri plates (1.5 × 6 cm diameter). The final concentration of paclitaxel in the agar medium ranged from 0.05 μg/ml (0.058 μM) to 4.0 μg/ml (1.52 μM). Plates that contained PDA with 1.5% ethanol or no ethanol served as the controls.

**Sensitivity of plant pathogenic fungi to the taxane fraction.** Isolates of 12 plant pathogenic fungi listed in Table 1 were cultured on PDA for 1-7 days prior to transferring them to the agar plates amended with the taxane fraction. Twenty-four hours after the amended plates were prepared, each plate was seeded on the perimeter with a 4-mm-diameter agar plug taken from the outer margin of an actively growing agar plate colonized by the test fungus. Petri dishes were sealed with parafilm and incubated at 24-26 C in the dark. Depending on the growth rate of the test fungus, the radial growths were measured to the nearest millimeter twice daily, daily, or every 3 days. The agar plug was not included in the measurement. Plates were monitored for 3 wk. The EC_{50} values (the effective taxane fraction concentration in the amended agar required to inhibit the growth of test fungus by 50% relative to the growth on the ethanol control plate) were computed by linear regression from plots of radial growth vs. concentration (19). The EC_{50} value was determined when the test organism on the ethanol control plate grew approximately halfway across the plate (2.5 cm from the edge of the agar plug). This time interval varied from 2 to 20 days, depending on the test fungus. The difference between the time required for the test fungus to grow 2.5 cm across the control plate vs. the time to grow 2.5 cm across the plate amended with taxane fraction containing paclitaxel at 4.0 μg/ml was also recorded. There were three plates for each.

**TABLE 1. The effective concentration (EC_{50}) required to inhibit some fungal species on taxane-fraction-amended agar**

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Taxonomic group</th>
<th>EC_{50}a (μg/ml)</th>
<th>Inhibition timeb (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>Deuteromycetes</td>
<td>&gt;4.00</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>Deuteromycetes</td>
<td>&gt;4.00</td>
<td>1.3</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Deuteromycetes</td>
<td>&gt;4.00</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Fusarium proliferatum</em></td>
<td>Deuteromycetes</td>
<td>&gt;4.00</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Verticillium dahliae</em></td>
<td>Deuteromycetes</td>
<td>&gt;4.00</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Venturia inaequalis</em></td>
<td>Ascomycetes</td>
<td>&gt;4.00</td>
<td>ND</td>
</tr>
<tr>
<td><em>Monilia fructicola</em></td>
<td>Ascomycetes</td>
<td>&gt;4.00</td>
<td>ND</td>
</tr>
<tr>
<td><em>Phytophthora cactorum</em></td>
<td>Oomycetes</td>
<td>0.50</td>
<td>ND</td>
</tr>
<tr>
<td><em>Phytophthora citricola</em></td>
<td>Oomycetes</td>
<td>0.50</td>
<td>16.0</td>
</tr>
<tr>
<td><em>Pythium aphidinenum</em></td>
<td>Oomycetes</td>
<td>0.05</td>
<td>ND</td>
</tr>
<tr>
<td><em>Pythium myriotylum</em></td>
<td>Oomycetes</td>
<td>0.80</td>
<td>ND</td>
</tr>
<tr>
<td><em>Pythium irregulare</em></td>
<td>Oomycetes</td>
<td>1.30</td>
<td>5.5</td>
</tr>
</tbody>
</table>

aEffective concentration of the taxane fraction (expressed as paclitaxel in μg/ml) required to inhibit the radial growth of the test fungus by 50%.

bDifference between the time required for the test fungus to grow to the approximate halfway point of the ethanol control plate and of the plate with the highest concentration of taxane fraction (4.0 μg/ml).

Values represent the mean of six plates. Values may be converted to μM paclitaxel by multiplying by 1.17.

Not determined; the radial growth on the 4.0-μg/ml plate at 26 days was 1.45 cm for *Verticillium dahliae* and *Venturia inaequalis, 0.9 cm for Phytophthora cactorum, 0.4 cm for *Pythium aphidinenum*, and 1.3 cm for *Pythium myriotylum*. 

1180 PHYTOPATHOLOGY
paclitaxel concentration, and the experiment was repeated. Standard errors were derived using data from both experiments.

Comparison of the taxane fraction to individual standards of authentic taxanes. To compare the relative toxicities of paclitaxel, cephalomannine, and baccatin III with the taxane fraction, the three taxane-sensitive species of *Pythium* were examined. The authentic compounds were dissolved in 95% ethanol and added to PDA to yield final concentrations of 0.1, 0.5, 1.0, and 2.0 μg/ml. The agar plates were prepared, seeded with *Pythium aphaniadermatum* (Edson) Fitz., *P. myriotyllum* Drechs., and *P. irregulare* Buulsman, and treated as described above. There were three plates of each fungus at each concentration of the taxane standards, and the experiment was repeated.

Comparison of the taxane fraction to a combination of authentic taxane standards. To determine whether taxane compounds other than paclitaxel, cephalomannine, and baccatin III in the extract contribute to the antifungal activity of the taxane fraction, an experiment was designed with the three species of *Pythium* to compare the taxane fraction to a combination of the authentic standards in the 10:5:1 molar proportion, respectively, which was typical in the taxane fraction. The agar plates were prepared, seeded with *P. aphaniadermatum*, *P. myriotyllum*, and *P. irregulare*, and treated as described above. There were three plates in this regime, and the experiment was repeated twice.

**RESULTS**

Extraction of taxanes. A typical liquid chromatogram (LC) trace of the taxane fraction from *Taxus* needles is shown in Figure 2. Several points are in order concerning these traces. First, the retention times of the paclitaxel and cephalomannine peaks identified in the trace of the taxane fraction were in agreement with those peaks in the LC trace of authentic standards (trace not shown). Second, the LC trace of the taxane fraction contained several peaks, presumably attributable to taxanes, in addition to paclitaxel and cephalomannine. Finally, the MetaChem Taxil column and the LC program employed permit resolution of the paclitaxel with *λ*~max~ at 230 nm from cinnamyl taxane analogs with *λ*~max~ at 280 nm (Fig. 2). These cinnamyl derivatives are known to co-elute with paclitaxel on C18 analytical HPLC columns and will therefore affect paclitaxel quantitation of the *Taxus* needle extracts performed on C18 columns (6). Further validation of the purity of the paclitaxel peak and hence of our quantification of the paclitaxel was evidenced in the UV spectra (Fig. 3) of the paclitaxel peak in the taxane fraction (A) and in the authentic standard (B), and of a peak assigned to a cinnamyl taxane (C).

Antifungal activity of the taxane fraction. Antifungal activity of the taxane fraction was assayed based on its paclitaxel concent-

![Fig. 2. Liquid chromatogram traces over time (min) at A, *λ* = 230 nm and B, *λ* = 280 nm of the partially purified taxane extracts (taxane fraction) from ornamental yews indicating the paclitaxel (Taxol) and cephalomannine peaks.](image)

![Fig. 3. UV spectra (absorbance) of paclitaxel (A), authentic paclitaxel (B), and the cinnamyl analogs (C) in the taxane fraction from ornamental yews.](image)
Comparison of the taxane fraction to paclitaxel, cephalomannine, and baccatin III standards. *P. aphanidermatum* and *P. myriotylum* were more sensitive to the taxane fraction than to equivalent concentrations of authentic paclitaxel, cephalomannine, and baccatin III (Table 2, Fig. 6A and B). Conversely, *P. irregularare* was more inhibited by authentic paclitaxel than by the taxane fraction (Fig. 6C). Cephalomannine had higher EC₅₀ values (i.e., was less toxic) than paclitaxel. Baccatin III was considered nontoxic to the three *Pythium* spp., and the EC₅₀ values could not be determined.

Comparison of the taxane fraction to a combination of the authentic taxane standards. When authentic paclitaxel, cephalomannine, and baccatin III were combined in 10:5:1 proportions, as is typical in the partially purified extracts, and compared to the taxane fraction, the radial growths of *P. aphanidermatum* and *P. myriotylum* were significantly more restricted on the agar amended with the taxane fraction containing paclitaxel at 1.0 µg/ml than on the agar amended with the same concentrations of taxane standards (Fig. 7A and B, Table 2). Once again, *P. irregularare* showed less sensitivity to the taxane fraction than to the combined authentic standards (Fig. 7C, Table 2).

**DISCUSSION**

Plant pathogens in the Oomycetes were ranked as highly sensitive to the taxane fraction in the in vitro studies reported here. The EC₅₀ values ranged from <0.05 to 1.30 µg/ml (0.058 to 1.52 µM). The few Ascomycetes and Deuteromycetes examined had minimal sensitivity to the taxane extraction. Lataste et al (13) reported that the ID₅₀ (the dose that will infect 50% of a tested population) of paclitaxel for disassembly of *Physarum polycephalum* microtubules was 0.9 µM. The EC₅₀ values for the five Oomycetes examined by Young et al (23) were 0.4–5.9 µM paclitaxel. Although the Oomycetes included in our study were not part of the studies of Lataste et al (13) or Young et al (23), the data from all three studies are in agreement despite the many differences among the assays. In the present study and the study by Young

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**Fig. 4.** Radial growth over time of *Pythium aphanidermatum*, *P. myriotylum*, and *P. irregularare* on potato-dextrose agar amended with the taxane fraction characterized by paclitaxel (Taxol) content (µg/ml). Control = ———, 0.05 = ———, 0.10 = ———, 0.50 = ———, 2.00 = ———, and 4.00 = ——— (not all concentrations are shown for each species); values represent the means of six radial growth measurements, standard error bars are included. *P. myriotylum* did not grow on agar containing taxane fraction at 4.0 µg/ml until the 18th day.

**Fig. 5.** The growth of *Pythium aphanidermatum* and *P. irregularare* on potato-dextrose agar amended with the partially purified taxane fraction characterized by the paclitaxel (Taxol) content (µg/ml).
et al (23), the EC₃₀ for the Deuteromycete Botrytis cinerea and the Ascomycete Monilinia fructicola were too high to be determined, even though the latter study included concentrations of paclitaxel greater than 40 μg/ml. Since our assay examined radial growth, which is not a measure of mycelial density or fungal weight, we believe the EC₃₀ values reported here underestimate fungal sensitivity to the taxane fraction. It is also possible that certain components of the taxane fraction are less available to fungi grown in agar.

Of all three authentic standards tested against P. aphanidermatum, P. myriotylum, and P. irregulare, paclitaxel was the most toxic, with EC₃₀ values of 0.10, 2.00, and 0.37 μg/ml, respectively. Cephalomannine was less toxic than paclitaxel but was still inhibitory to two of the three Pythium species. Since paclitaxel was more toxic than cephalomannine and was present typically in the taxane fraction at twice the concentration, it is logical to assume that paclitaxel is the major contributor to the toxicity of the taxane fraction. Likewise, the absence of toxicity of baccatin III and its low concentration in the taxane fraction indicate that it contributed little to the activity of the taxane fraction. However, the inclusion of paclitaxel and cephalomannine in the extract did not explain why P. aphanidermatum and P. myriotylum were more restricted by the taxane fraction at 1.0 μg/ml than by the three standard mixtures containing the same concentrations. Indeed, for P. myriotylum, we provided persuasive evidence (Fig. 7B) that other compounds in the extract, presumably taxanes, contributed additively and/or synergistically, making it more effective than a combination of authentic standards. The greater sensitivity of P. irregulare to the authentic paclitaxel standard and to the combined preparation of the three authentic standards than to the taxane fraction is unexplained. It is possible that other taxanes in the extract compete with paclitaxel for the active site on the tubulin of P. irregulare or for transport sites across the cell membrane.

As unique mitotic poisons, taxanes prevent the depolymerization of microtubules. Latasa et al (13) reported that paclitaxel acted similarly on mammalian tubulin from pig brain and tubulin from the slime mold Physarum polycephalum at roughly the same

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**Fig. 6.** Radial growth of Pythium aphanidermatum, P. myriotylum, and P. irregulare after 3 days on potato-dextrose agar amended with different concentrations (μg/ml) of the taxane fraction characterized by the paclitaxel (Taxol) content (●), authentic paclitaxel (△), cephalomannine (○), or baccatin III (▲); values represent the means of six radial growth measurements; standard errors were computed, but error bars were usually smaller than the symbols and are not shown.

**Fig. 7.** Radial growth of Pythium aphanidermatum, P. myriotylum, and P. irregulare after 3 days on potato-dextrose agar amended with the taxane fraction characterized by paclitaxel (Taxol) concentration (●) or a combined standard of authentic paclitaxel, cephalomannine, and baccatin III in a 10:5:1 concentration ratio, respectively (○); values represent the means of six radial growth measurements; standard errors were computed, but error bars were usually smaller than the symbols and are not shown.
concentrations (0.4–0.8 μg/ml), leading them to suggest that the tubulin target site of interaction with taxanes may have been conserved in these evolutionarily divergent eukaryotes. On the other hand, we found wide differences in taxane sensitivity among plant pathogens from presumably different evolutionary origins. Furthermore, although the mammalian β-tubulin subunit has been suggested as the binding site with paclitaxel (16), Cabral et al. (5) found paclitaxel resistance in a mutant from Chinese hamster ovary cells that resulted from an alteration on the α-tubulin monomer. Investigations into the mechanisms of taxane cytotoxicity and effective concentrations across species are well warranted.

The antiphenolic fungicidal compounds carbendazim and nociocazole act as mitotic poisons by preventing the assembly of tubulin into microtubules, and their site of action is reported to be the β-tubulin subunit (7). Although both compounds are active against Ascomycetes and Deuteromycetes, only nociocazole is effective against Oomycetes, higher plants, and mammalian cells (9). Given the findings that a specific site on tubulin confers sensitivity to the taxanes, our data suggest that tubulin belonging to the Ascomycetes and Deuteromycetes may differ dramatically in composition from Oomycetes tubulin. In accordance with this observation, recent advances in fungal systematics indicate a distinctly separate evolutionary origin for the Oomycetes than for the Ascomycetes and Deuteromycetes (3). It is also possible that detoxification mechanisms are operative. Young et al. (23) demonstrated that fungal sensitivity was reduced or completely lost when the biologically active side chain on the taxane A ring was shortened or removed (Fig. 1). Exposing this side chain would produce the biologically less active taxane analog, baccatin III, which at concentrations of 2.0 μg/ml was not toxic to our most sensitive taxane fungus, P. aphidermatum (Fig. 6A). Lataste et al. (13) reported that baccatin III was very active in binding to microtubules of Physarum polycephalum in vitro, but the authors (22) reported that only paclitaxel was included in vivo cell division in the amoebal stage of this organism. The taxane-insensitive species may possess an enzyme for converting biologically active taxanes to inactive taxanes. Since the Deuteromycete B. cinerea and the Ascomycete M. fructicola were completely uninhibited by the taxanes in our study (EC50 > 4.0 μg/ml) and in those of Young et al. (23) (>43.0 μg/ml), we have initiated studies to determine whether these fungi are capable of converting paclitaxel to baccatin III.

The selectivity of these taxanes to Oomycetes is intriguing and may offer a new chemical tool for selectively inhibiting these plant pathogens. Depending on the pathogen, a synergistic antifungal effect is possible with the taxane fraction. It is possible that the taxane fraction also has a broader spectrum of fungicidal efficacy than pure standards. More importantly, the development of pure paclitaxel for use as an agrochemical is unrealistic, because of both the urgent demand for its application as a chemotherapeutic anticancer agent and the great expense of obtaining pure quantities from yews. Further exploration of the unique mode of action of the economically accessible taxane fraction (obtainable from a waste product of nursery operations) against the Oomycetes is clearly warranted.

LITERATURE CITED


