Effect of Taxol and Related Compounds on Growth of Plant Pathogenic Fungi

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ABSTRACT

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Taxol, currently in use as therapy for refractory ovarian cancer, has no known function in the plant (*Taxus brevifolia*) from which it is isolated. In an effort to explore its biological role, we investigated the effect of Taxol and related compounds on the growth of plant pathogenic fungal hyphae. Taxol and cephalomannine inhibited the growth of several fungi, especially *Phytophthora* and *Pythium* species (Oomycetes) and *Rhizoctonia solani* (Basidiomycetes). Baccatin III had no effect on the growth of any fungus tested. Strains of *Aspergillus* (Deuteromycetes) and *Fusarium* (Ascomycetes) were not inhibited by any taxane. As little as 1.0 nmol of Taxol or cephalomannine severely retarded radial growth of *Phytophthora* species on solid medium. A dose-dependent effect was also evident in liquid culture assays down to 0.1 μM Taxol, with total inhibition of mycelial growth at 1.0 μM. At lower concentrations of Taxol, the fungal mycelium reached stationary stage 4 days later than the controls, indicating growth retardation rather than death. When hyphae were transferred to Taxol-free media, growth resumed as normal. Active doses are roughly 600 times lower than an estimated concentration of Taxol in *Taxus* needles. It is proposed that Taxol may be a preformed antifungal defense compound in *Taxus* species.

The Pacific yew has been used by Native Americans in the Pacific Northwest as a source of tools, weapons, and sedative tonics for hundreds of years (15). Taxol (paclitaxel) is one of a group of diterpene amides, known as taxanes, that are produced by the Pacific yew (*Taxus brevifolia* Nutt.) (31) and other members of the *Taxus* genus (33). It is currently being developed as a cancer drug because of its potent and relatively specific activity (23). A variety of cells (34) and many human cancers, including leukemia and certain breast, ovarian, and lung cancers (21,31), are affected by Taxol. The efficacy of this unique molecule stems from its ability to promote microtubule formation and inhibit postmitotic spindle disassembly (13,26). This is in contrast to other mitotic poisons, such as benomyl and griseofulvin, which act as microtubule assembly inhibitors. Benzimidazole fungicides act by binding directly to tubulin (7), while griseofulvin binds to microtubule-associated proteins (MAPs) (22).

Although Taxol's mode of action on mammalian cells is well established, little is known about its biological function in plants. Taxol content varies widely among individuals and populations of yew trees (32). Reported averages range from 0.001 to 0.01% (w/w) for dry needles and stems (33), and up to 0.01% (w/w) for roots (29). Taxol is found throughout the plant along with a wide number of endophytic microorganisms, including potential pathogens (25). Recently, a new species of fungus (*Taxomyces andreanae*) with reported Taxol-synthesizing ability was isolated from yew bark (28). It has been suggested that Taxol may serve as a defense compound, retarding the growth of invading microorganisms and fungi (30). A fungal root rot caused by *Phytophthora lateralis* has been reported to cause the death of *T. brevifolia* found in close proximity to infected Port-Orford cedar (*Chamaecyparis lawsoniana*), the primary host (3,14). Other *Phytophthora* species are pathogenic to the cultivated yew species *Taxus* × *media* and *T. baccata*, causing severe losses of cuttings in greenhouses. Species of the Oomycete family are resistant to widely used fungicides, such as griseofulvin and benalate derivatives. Based on this information, we tested the action of Taxol, cephalomannine, and baccatin III (Taxol derivatives) against fungal strains known to infect members of the genus *Taxus*, as well as against some common root pathogens.

MATERIALS AND METHODS

Fungal culture. Cultures were obtained from ATCC (*Aspergillus fumigatus*, 26933; *Fusarium oxysporum*, 52359; *Phytophthora lateralis*, 56876), Louisiana State University (*Phytophthora drechsleri, Pythium polytylum, Pythium ultimum, Phycomyces blakesleeanus, Rhizoctonia solani*), Pennsylvania Department of Agriculture in Harrisburg (*Phytophthora cinnamomi*, 71036A-88 A2), the Connecticut Agricultural Experiment Station (*Phytophthora citrophthora, Phytophthora palmivora, Pythium myriotylum, Pythium irregulare*), and the Plant Pathology Department at Pennsylvania State University (*Aspergillus nidulans*). Cultures were maintained on solid potato-dextrose agar in the dark at 25 C and subcultured monthly. Assays were carried out with mycelia grown on Schenk and Hildebrandt's medium modified for fungal culture (8) and solidified with Gelrite at 3.5 g/L and 0.4 g of $MgSO_4 \cdot 7H_2O$. This medium was used because its components are defined and it is completely clear, making the hyphal growth easy to measure.

Growth assays. The biological activity assays were performed following a protocol modified from Roberts and Selitrennikoff (21). Mycelial plugs (3 mm diameter) from actively growing plates were used to inoculate test plates and flasks. Test plates consisted of actively growing plugs of mycelium surrounded by sterile filter paper wedges (3-cm disks cut in quarters) soaked with standard stock solutions of purified baccatin III, cephalomannine, and Taxol (together referred to as taxanes and provided by K. Snader

of the National Cancer Institute). Inhibition was qualitatively defined as a mycelium-free zone surrounding a wedge. Stock solutions of taxane standards and dried needle extracts were dissolved in dimethyl sulfoxide (DMSO). Controls consisted of dry disks and disks wetted with DMSO alone. Growth inhibition assays were also performed in liquid culture. Assays were carried out in 50-ml Erlenmeyer flasks incubated in the dark at 25 C on a rotary shaker (80 rpm) and in 125-ml standing flasks, both with 20 ml of medium. Controls included flasks containing medium alone and flasks with an amount of DMSO equivalent to that used to deliver the highest taxane dose. Controls for the extract assay also included medium containing extract that had been filtered through a 2-μm filter (Gelman Sciences, Inc., Ann Arbor, MI). Flasks were harvested at regular intervals or at the end of the growth phase (12–14 days for most fungi). Mycelia were blotted with paper towels and weighed, then dried to a constant weight in an 80 C oven. Inhibition of growth was defined as a reduction in dry weight compared to the controls. Three or four replicates per treatment were included, and each experiment was repeated at least once. Student's t test was performed using Lotus 1-2-3 software.

To determine the nature of the inhibition, samples of mycelium from 2-wk-old inhibited and control cultures were transferred to Taxol-free medium. Growth of these cultures was measured as an increase in diameter over time.

Taxane extraction and high-pressure liquid chromatography (HPLC) analysis. Taxanes were extracted from needles of *Taxus* × *media* 'Hicksi' following the method of Mattina and Paiva (19). Fresh needles were ground in a blender with MeOH at a ratio of 10 g of needles:100 ml of MeOH. The slurry was incubated on a rotary shaker for >9 h and filtered through Whatman No. 4 qualitative filter paper. The extract was evaporated to dryness, redissolved in 11 ml of 10:1 MeOH:H_2O for every 10 g of original tissue, and loaded in 1-ml aliquots onto a C18 solid phase extraction column (J. T. Baker, Inc., Phillipsburg, NJ) equilibrated with H_2O. The column was washed with H_2O, then with 10 ml each of 20, 50, and 80% MeOH. The 80% MeOH fractions were collected, pooled, and evaporated to dryness. The residue was redissolved in 1 ml of MeOH for quantification of Taxol content by HPLC (10). Instrumentation consisted of a Waters 600E System equipped with a U6K injector and 990 Photodiode Array detector (Millipore Corporation, Waters Chromatography Division, Milford, MA). Samples were separated by a 250 × 4.6 mm Curosil G (4-μm particle size) column (Phenomenex, Torrance, CA) and eluted using a linear gradient of 10 mM ammonium acetate (pH 4.0):acetonitrile that changed from 65:35 to 45:55 in 20 min. These conditions were held for 17 min before returning to the initial solvent mix and equilibrating for 15 min. Identification and quantification of taxanes in tissue extracts was based on comparison of peak retention time in at least two different gradient systems: UV (220–290 nm) absorbance spectra, and spiking of the samples with standards. UV absorbance spectra and absorbance ratios of the putative peaks at 280/230 nm corresponded to the purified standard supplied by the NCI. This information was used for activity comparisons between needle extracts and the pure Taxol standard. The extract was evaporated, and the residue was dissolved in an appropriate amount of DMSO for use in the liquid growth assay.

Medium extraction. Medium from a time course experiment with Taxol and *Phytophthora cinnamomi* was assayed for taxane

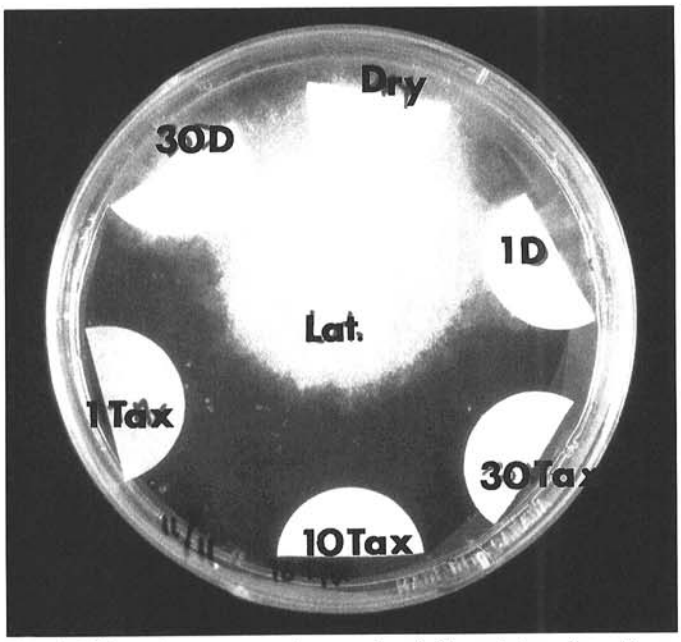

Fig. 1. Effect of taxanes on the growth of *Phytophthora lateralis* on solid medium. A 3-mm plug of mycelium was placed in the center of six sterilized filter paper wedges each containing, clockwise from the top, Dry = dry control, 1D = 1 μl of dimethyl sulfoxide (DMSO), 30Tax = 30 nmol Taxol, 10Tax = 10 nmol Taxol, 1Tax = 1 nmol Taxol (delivered in DMSO), and 30D = 30 ul DMSO. Inhibition was qualitatively determined by the size of the zone of inhibition surrounding the test wedge. All later plate assays were carried out in this manner.

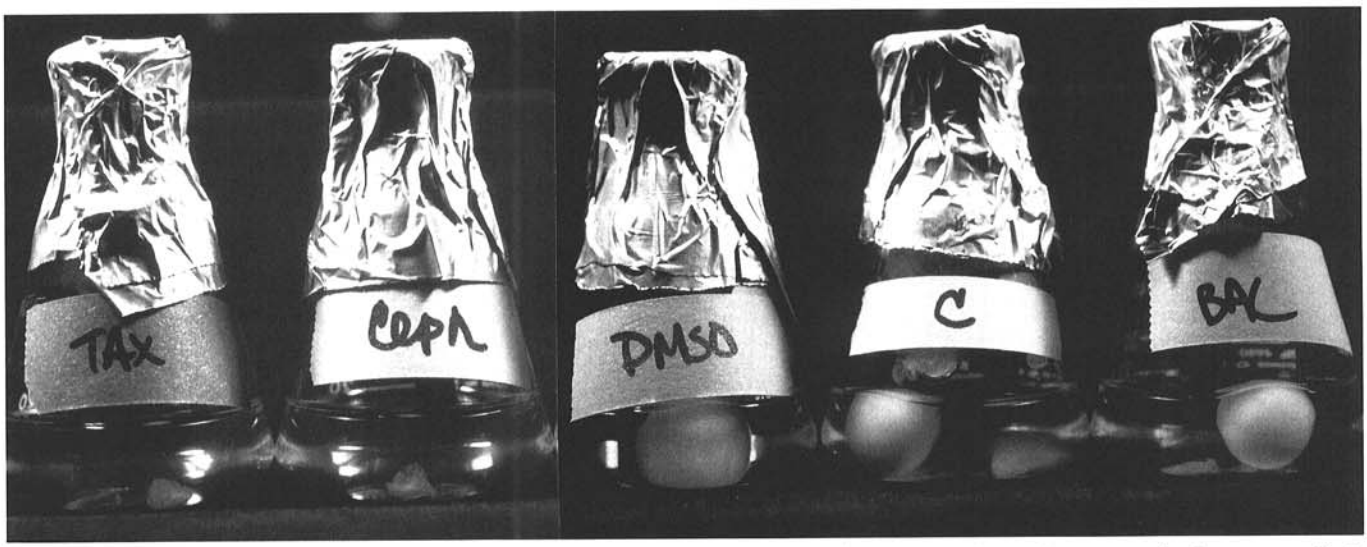

Fig. 2. Effect of taxanes on the growth of *Phytophthora lateralis* in liquid medium. The inoculum used was the same as in plate assays. Flasks contain 10 μM taxanes (Bacc = baccatin III, Ceph = cephalomannine, and Tax = Taxol), 10 μl dimethyl sulfoxide (DMSO), or plain medium (C). Growth was completely inhibited in the Taxol and cephalomannine flasks.

content. The media were lyophilized, extracted in CH_3Cl, clarified by centrifugation, and evaporated to dryness. The residue was redissolved in an appropriate volume of 50:50 H_2O:CH_3CN and centrifuged to remove particulates. Samples were then analyzed by HPLC as above (11).

RESULTS

Growth inhibition by taxanes. Initial experiments were designed to determine if taxanes had any effect on fungal growth. Both Taxol and cephalomannine were very effective in retarding the growth of *Phytophthora lateralis* on plates and in liquid culture (Figs. 1 and 2). However, the taxane solvent, DMSO, also significantly retarded growth (Fig. 3A). Dimethyl sulfoxide had no significant effect on any other fungus tested on plates or in liquid culture (Figs. 3 and 4). *Phytophthora lateralis* grows very slowly, which may explain its high sensitivity to Taxol and cephalomannine compared to other members of the same genus (Figs. 1 and 2A). The slow growth rate may allow more time for the Taxol to diffuse into the tissue.

Fungi of the same genus and others of the Oomycete class also showed sensitivity to Taxol and cephalomannine (Table 1, Fig. 3). Members of other classes, including the Basidiomycetes, Deuteromycetes, and Ascomycetes, showed limited sensitivity, if any (Table 1). The fast-growing fungi *R. solani* (Fig. 3D) and *Pythium aphanidermatum* (Table 1) showed no response on culture plates but were sensitive in liquid assays. Baccatin III, a proposed precursor of Taxol that lacks the C13 side chain, had no apparent effect on any fungus tested (Fig. 3). Apparently the side chain, even if modified as in cephalomannine, is necessary for antifungal activity (34). Interestingly, the C13 side chain is also necessary for anticancer activity (13).

The response of *Phytophthora cinnamomi* was representative of other sensitive fungi (Fig. 3C), so the inhibitory activity of Taxol on this fungus was investigated further. The difference between Taxol and cephalomannine activity was not statistically

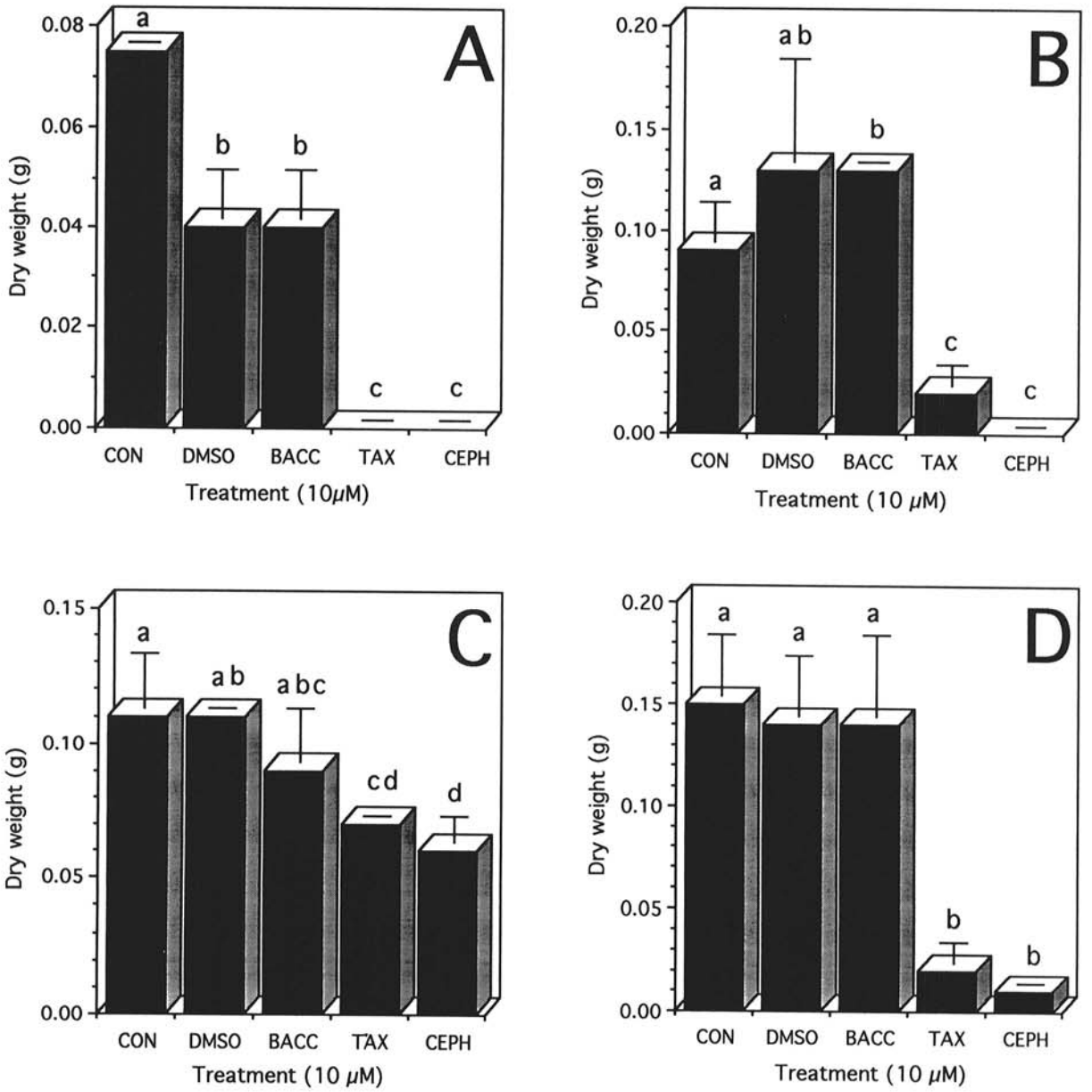

Fig. 3. Effect of taxanes on a sampling of plant pathogenic fungi. **A,** *Phytophthora lateralis,* a root pathogen reported to kill *Taxus brevifolia* in the wild (14); **B,** *Phytophthora drechsleri* and **C,** *Phytophthora cinnamomi,* common greenhouse pathogens; and **D,** *Rhizoctonia solani,* an opportunistic fungus, was the least susceptible of fungi showing an effect from taxane treatment. Based on this data, *P. cinnamomi* was used as the model for dose response assays and morphology studies. CON = control or no treatment, DMSO = dimethyl sulfoxide, BACC = baccatin III, TAX = Taxol, and CEPH = cephalomannine. Bars represent one standard deviation. Columns with the same letter are not significantly different ($P = 0.05$ [*R. solani,* $P = 0.10$]).

significant ($P = 0.05$) in any species tested (Fig. 3); therefore, only Taxol data will be reported hereafter.

Dose response. The effect of Taxol on the growth of *Phytophthora* spp. is dose dependent. Concentrations of 1 μM and greater severely inhibit growth over 1 wk. Lower concentrations resulted in limited growth compared to controls (Fig. 4). Taxol concentrations of 0.1 and 0.5 μM change the shape of the *Phytophthora cinnamomi* sigmoidal growth curve; the lag and log phases are extended, and the stationary phase is delayed (Fig. 5). However, the final dry weight of the cultures, regardless of treatment, equilibrated over time (Fig. 5). Apparently, low concentrations of Taxol may allow limited growth of the fungus without killing the mycelium.

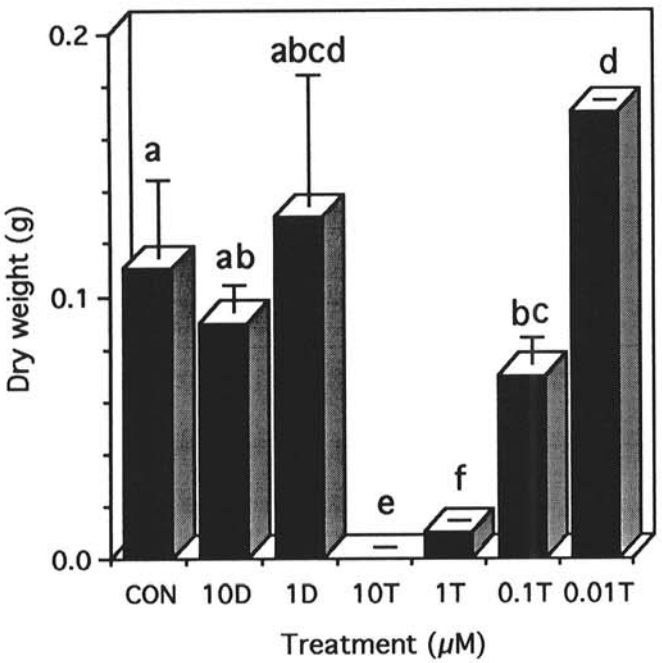

Fig. 4. Dose response of *Phytophthora cinnamomi* to varying concentrations of Taxol in liquid culture. CON = no treatment, 10D = 10 μl dimethyl sulfoxide (DMSO), 1D = 1 μl DMSO, 10T = 10 μM Taxol, 1T = 1.0 μM Taxol, 0.1T = 0.1 μM Taxol, and 0.01T = 0.01 μM Taxol. The minimum inhibitory dose was determined to be 0.1 μM. Bars represent one standard deviation. Columns with the same letter are not significantly different ($P = 0.05$).

TABLE 1. Effect of Taxol on the growth of fungi in plate and liquid assays. Not all fungi were tested in both assays

Species	Test	Activity	MID[a]
Aspergillus fumigatus	Plate	No	ND[b]
Aspergillus nidulans	Plate	No	ND
Fusarium oxysporum	Flask	No	ND
Phycomyces blakesleeanus	Flask	No	ND
Phytophthora cinnamomi	Flask	Yes	0.1 μM
Phytophthora citrophthora	Plate	Yes	1.0 nmol
Phytophthora drechsleri	Flask	Yes	0.1 μM
Phytophthora lateralis	Plate	Yes	1.0 nmol
	Flask	Yes	0.1 μM
Phytophthora palmivora	Plate	Yes	1.0 nmol
Pythium aphanidermatum	Plate	No	ND
	Flask	Yes	0.1 μM
Pythium myriotylum	Plate	Yes	1.0 nmol
Pythium polytylum	Plate	Yes	1.0 nmol
Pythium irregulare	Plate	Yes	20 nmol
Pythium ultimum	Plate	Yes	1.0 nmol
Rhizoctonia solani	Plate	No	ND
	Flask	Yes	10 μM

[a]Minimum inhibitory dose = the lowest concentration that showed a significant decrease in growth compared to controls.
[b]Not determined.

To determine if this effect is due to a depletion of Taxol in the medium over time, new hyphal plugs were inoculated into conditioned control and Taxol-containing media. The conditioned medium was taken from the last time point in the growth inhibition experiment represented in Figure 5. The fresh inocula showed no increase in dry weight after 2 wk of culture. This may indicate that both the control and the 0.1 μM Taxol medium, in which the previous culture had reached stationary phase (Fig. 5), were nutritionally depleted. However, the mycelium in the 0.5 μM treatment initial culture was in log phase when harvested (Fig. 5), showing that the medium was not nutritionally depleted and that Taxol may be present in inhibitory levels. Analysis of the medium by HPLC before and after growth experiments showed no change in Taxol content between fresh and conditioned medium (data not shown). In addition, fungal hyphae incubated with 0.5 μM Taxol for 3 wk grew normally once they were placed on fresh Taxol-free medium. These results suggest that Taxol is not metabolized by or irreversibly bound to the mycelium.

Extract assays. An extract of *Taxus* needles was added to basal medium based on Taxol content as determined by HPLC. These aliquots were dispensed as Taxol equivalents where one μequivalent contains one μg of Taxol plus other compounds found in the needle extract. Growth inhibition of *Phytophthora cinnamomi* in liquid culture occurred at 1.0 μequivalents/ml (1.17 μM) (Fig. 6). Flasks containing a corresponding amount of pure Taxol (1.0 μg/ml = 1.17 μM) did not grow (Fig. 6). During initial experiments with concentrated needle extracts, some precipitation occurred when extracts were added to the medium. As a control, medium containing 2.5 μequivalents/ml was filtered through a 0.2 μm filter before addition of the fungal inoculum. This treatment did not significantly change the inhibitory effect at $P = 0.05$ (Fig. 6). While needle extracts did significantly decrease the growth of *Phytophthora cinnamomi,* the minimum inhibitory dose was 10 times greater than that of purified Taxol, suggesting there may be compounds in the extract which inhibit Taxol activity.

DISCUSSION

There has been some speculation on the evolutionary advantage of producing a molecule like Taxol, which has a unique mode

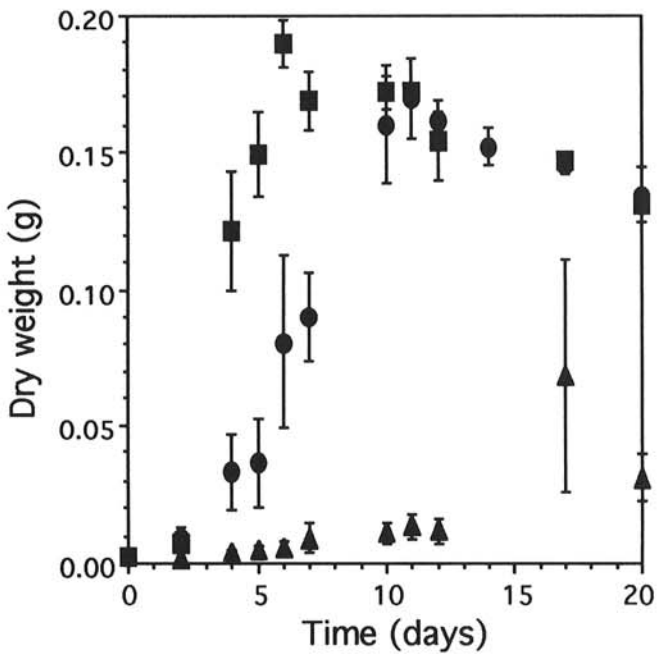

Fig. 5. Time course of *Phytophthora cinnamomi* growth when under the influence of Taxol. ■ = control, ● = 0.1 μM Taxol, and ▲ = 0.5 μM Taxol. Cultures treated with Taxol at sublethal doses grew slowly and eventually reached stationary phase. Bars represent one standard deviation.

of action on the microtubules of mammalian cells. In contrast to typical microtubule assembly inhibitors such as griseofulvin and benomyl, Taxol stabilizes microtubules against depolymerization and effectively freezes the cell in mitosis (26,34). A wide variety of eukaryotic cells are affected by Taxol, including *Xenopus* and sea urchin eggs (16,24), *Haemanthus* endosperm (1), *Poterioochromonas* (17), and *Trypanosoma* (2). A recent paper by Young et al (34) showed that Taxol is active on a variety of fungi from the Oomycete, Basidiomycete, Deuteromycete, and Ascomycete classes in poison agar assays. Of these groups, only the Oomycetes were highly susceptible, with EC_{50}s on Taxol-containing solid medium of less than 6 μM (34). Only a few plant-pathogenic fungi were studied in that report. Our data show that species of *Phytophthora* and *Pythium,* both Oomycetes, were susceptible to 0.1 μM Taxol in liquid medium (Table 1). Also, *R. solani* and *Pythium aphanidermatum* were relatively insensitive to Taxol on solid medium but sensitive when it was presented in a liquid environment.

The minimum inhibitory dose for *Phytophthora cinnamomi* in liquid culture was determined to be 0.1 μM (Fig. 4). At this level, hyphae grew more slowly than the control but did eventually reach the full volume of the contol at stationary phase (Fig. 5). At 0.5 μM, growth was even slower, with a more rapid rate occurring near the end of the assay period. These data, taken together with the fact that the medium showed no change in Taxol content before and after the growth of the fungi, lead us to conclude that Taxol is a nonmetabolizable fungistat. This is consistent with the resumption of hyphal growth observed after the mycelium was transferred to Taxol-free medium.

These low active concentrations were compared to reported values of the Taxol concentration in needles of live yew. We used needles as a model because Taxol content data for needles are more available (3,19,29,31–33) and reliable than root data (29), and we wanted to compare our calculated estimate to growth experiments with needle extracts. Information on Taxol content is commonly reported as percent or parts per million (ppm) dry weight; therefore, we converted this unit into micromoles in order to compare our in vitro results with fungi to concentrations reported in the literature. We based our calculations on the conservative assumptions that needles contain between 0.01 and 0.04% (100–400 ppm) dry weight Taxol, on average (19), and have a fresh weight to dry weight ratio of 8.0. This ratio is higher than both the reported value of 2.5 for winter-sampled tissue (19) and 3.7 for greenhouse samples (L. J. Wagner, 1994, *unpublished*), but on the lowest extreme of fully hydrated tissue culture samples (8–20)(6). The tissue culture value more accurately reflects the water content of an actual cell, while the needle values include the thick, waxy cuticle. A biotic volume of 1.02 ml, based on the specific gravity of plant cells (27), was used to include the lipid volume of the cell, a possible site of Taxol localization. The concentration of Taxol within a cell was therefore determined to be between 15 and 60 μM. This very conservative estimate is at least 150 times greater than the minimum inhibitory dose found in liquid culture. This calculation did not account for any sequestering or localization, so the subcellular concentrations may actually be higher. It is therefore conceivable that Taxol and other taxanes are biologically relevant molecules in the pathogen defense strategies of *Taxus* spp.

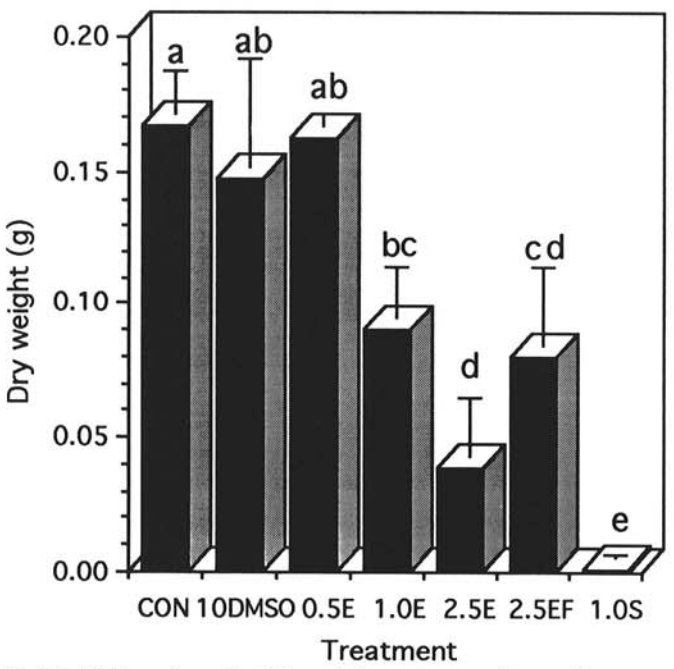

Fig. 6. Inhibition of growth of *Phytophthora cinnamomi* by needle extracts applied at biologically relevant concentrations. CON = no treatment, 10DMSO = 10 μl dimethyl sulfoxide, 0.5E = 0.5 μequivalents/ml medium, 1.0E = 1.0 μequivalents/ml medium, 2.5E = 2.5 μequivalents/ml medium, 2.5EF = same as 2.5E but passed through 2-μm filter after addition of extract, and 1.0S = pure Taxol at 1.0 μg/ml medium (1.17 μM Taxol). One μequivalent, used to measure needle extracts, contains one μg of Taxol plus the other compounds found in the extract. All treatments were delivered in DMSO. Bars represent one standard deviation. Columns with the same letter are not significantly different ($P = 0.05$).

In support of this hypothesis, needle extracts from *T.* × *media* 'Hicksi' did inhibit the growth of *Phytophthora cinnamomi,* although 10 times less strongly than pure Taxol (Fig. 6). The minimum inhibitory dose of our extract was in the range of that reported by Elmer et al (9), who found that as little as 0.5 μequivalents/ml (0.59 μM) inhibited the growth of *Phytophthora cactorum* in poison agar assays. These figures are all within range of each other and well under the calculated concentration of Taxol in needle tissues.

Because of the coenocytic nature of the Oomycetes, the inhibition of growth cannot be due to prevention of septate wall formation. Mitosis is endonuclear, and hyphae grow from the tip by vesicle transport (12,18). Young et al (34) reported an inhibition of nuclear division by Taxol in germinating zoospores of *Phytophthora capsici* that preceded the negative effect on germ tube elongation. However, the dose of Taxol used (8.0 μM) was five times greater than the EC_{50} of 1.6 μM. Observation of *Phytophthora cinnamomi* treated with 0.1 or 0.5 μM Taxol and stained with DAPI to show nuclei under Nomarski optics (DIC) did not reveal any obvious differences in nuclei content when compared to controls (L. J. Wagner, 1994, *unpublished*). These doses are much lower than previously reported and do slow growth (Fig. 3) and increase the frequency of branching (L. J. Wagner, 1994, *unpublished*). We propose that Taxol may inhibit vesicle transport along microtubules and affect growth at much lower concentrations than those needed to affect endonuclear mitosis. Current experiments are focusing on the immuno-localization of microtubules in normal and inhibited hyphae of *Phytophthora cinnamomi* in an effort to characterize this effect (30).

In conclusion, the above information suggests a possible role for taxanes in the defense mechanisms of *Taxus* spp. Many gymnosperm species, such as pines, produce terpene compounds in response to wounding and pathogen attack (5). Investigation of taxane and other terpene effects on plant pathogenic fungi may provide leads to novel biofungicides. This approach could also be used to enhance Taxol production in vitro through the use of elicitors (4,10). Analysis of different species of fungi that are susceptible and resistant to Taxol as a growth inhibitor may also provide leads to new uses for taxanes in human or animal drug therapy (20).

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