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Production of antifungal metabolites by the ectomycorrhizal fungus *Pisolithus tinctorius* strain SMF

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SUMMARY

An ectomycorrhizal fungus, *Pisolithus tinctorius* strain SMF, isolated from a basidiocarp removed from the roots of a recently fallen old growth fir in the Smoky Mountains of Tennessee, was characterized for its in vitro production of antifungal metabolites. On solid medium *P. tinctorius* SMF strongly inhibited growth of strains of *Fusarium solani*, *Geotrichum candidum*, *Phanerochaete chrysosporium*, and *Verticillium dahliae*, all species known to be plant pathogens. Evidence from paired colony growth inhibition studies on agar plates indicated that production of antifungal agents by *P. tinctorius* SMF may be enhanced by close physical contact with other fungi. The antifungal activity of *P. tinctorius* SMF was much greater than that of several culture collection strains of *P. tinctorius*. The culture collection strains either showed no or very limited activity. The antifungal activity was associated with an apparently inducible metabolism of *P. tinctorius* SMF and with the production of darkly colored water soluble phenolic metabolites. Small scale fermentation studies showed that the phenolics are readily producible by submerged culture fermentation. This is the first report of submerged culture production of antifungal metabolites by an ectomycorrhizal fungus.

INTRODUCTION

The ectomycorrhizal fungus *Pisolithus tinctorius* occurs in 33 countries and in 38 states in the United States, and it can form ectomycorrhizae with a wide range of hosts in various environments [13]. Ectomycorrhizal fungi benefit their host tree by increasing nutrient and water absorption and by increasing tolerances to stresses such as drought, extreme soil temperature, extreme soil pH, low fertility, and high concentrations of heavy metals [16]. The presence of ectomycorrhizal fungi, such as *P. tinctorius*, has repeatedly been shown to protect tree roots against invasion by root-pathogenic soil fungi [7]. The use of pure cultures of *P. tinctorius* to inoculate tree seedlings has been reported to improve survival and growth [12,15,16]. Root protection against soil-borne plant pathogenic microorganisms, often termed microbial antagonism, may result from the production of antifungal chemicals, antibiotics, and/or extracellular hydrolytic enzymes by root-inhabiting ectomycorrhizal fungi [1,4,8,10,14,18]. Protection may not only be effective inside the root, but probably also extends into the rhizoplane or rhizosphere [3]. Mycorrhizae-associated terpenes and sesquiterpenes have been shown to inhibit root pathogenic fungi [9]. In vitro synthesis of antifungal compounds has been reported for several pure cultures of ectomycorrhizal fungi [2,11,17,18,19], including recently *P. tinctorius* [8]. However, fermentative production of antifungal metabolites by ectomycorrhizal fungi has not yet been reported to our knowledge.

We cultured an ectomycorrhizal fungus identified as *P. tinctorius* SMF, from a basidiocarp collected from a fir tree in the Smoky Mountains near Gatlinburg, Tennessee. Here, we report on the phenolic-associated antifungal activity of *P. tinctorius* SMF towards selected fungal species known to be soil-borne plant pathogens, and we present data to show that these metabolites can be readily produced by *P. tinctorius* SMF in submerged culture fermentation.

MATERIALS AND METHODS

Microorganisms

(a) Fungal strains. P. tinctorius SMF was cultured from a basidiocarp collected from the roots of a recently fallen fir tree in the Smoky Mountains near Gatlinburg, TN, U.S.A. Basidiospores and possibly peridote fungal matrix,

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removed from the basidiocarps, were incubated on potato dextrose agar (PDA) plates (pH 5.6–5.7) at 25 °C in darkness. Strain SMF was obtained from a single fungal colony that grew up on a PDA plate and maintained on PDA slants. Three culture collection strains of *Pisolithus tinctorius*: strains 471, 370, and 359 (from the Department of Forest Science, at Oregon State University, Corvallis, OR), and four common soil-borne fungal pathogens: *Fusarium solani*, *Phanerochaete chrysosporium*, *Geotrichum candidum*, and *Verticillium dahliae* (from Dr. J.B. Sutherland, FDA National Center for Toxicology Research, Jefferson, AR), were maintained on PDA plates. These cultures were grown at 25 °C in darkness. These four strains were identified as 'pathogens' when obtained, but were not retested for their pathogenicity.

(b) Identification of P. tinctorius SMF. Mycelial squares (7–10 mm) of *P. tinctorius* SMF or specific culture collection strains previously grown on PDA plates, were transferred aseptically to a 100-ml baffled flask containing 25 ml of further modified Melin-Norkrans (FMMN) liquid medium (pH 5.7), which was slightly modified from MMN [10], and incubated shaking at 120 rpm for 2 weeks. The FMMN contained, per 1.0 liter distilled water: 3.0 g Malt Extract (Difco), 10.0 g dextrose, 0.25 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7\,H_2O$, 0.05 g $CaCl_2$ (all from J.T. Baker), 0.05 g NaCl (EM Science, Cherry Hill, NJ), 1.0 ml FeEDTA Solution, and 1.0 ml vitamin solution. The FeEDTA solution contained 3.36 g Na_2EDTA (Sigma Chemical Co.), and 2.78 g $FeSO_4 \cdot 7\,H_2O$ (J.T. Baker) in 100 ml distilled water, while the vitamin solution contained 0.1 g nicotinic acid, 1.0 g thiamine HCl, 10 g *myo*-inositol (all from Sigma), and 0.1 g pyridoxine · HCl (Nutritional Biochemicals Corporation) in 100 ml distilled water. The initial pH was adjusted to 5.5–5.7 and the carbon source was sterilized separately. After 2 weeks, *P. tinctorius* SMF and each culture collection strain were compared to each other by fluorescence microscopy (Zeiss standard fluorescence microscope system, North Central Instruments, Minneapolis, MN). Mycelial growth and morphology of each strain were compared. Cultures were microscopically examined periodically to confirm their purity.

Paired colony growth antagonism studies on PDA plates

F. solani, *P. chrysosporium*, *G. candidum*, and *V. dahliae*, common soil-borne plant pathogenic fungi of conifers such as pine and Douglas-Fir, were grown on PDA plates. The first three were incubated for 8–12 days and the last one for 24–30 days at 25 °C in darkness. Three similar mycelial squares bearing conidial spores of each test fungus were placed between the *P. tinctorius* SMF colonies that had grown for six days on the plates. Growth rates of both *P. tinctorius* SMF and each fungus were then monitored periodically by measuring colony diameters. Antagonism of *P. tinctorius* SMF towards the fungi was determined by the difference of the growth rates of the pathogens on plates also containing *P. tinctorius* SMF, as compared to plates inoculated with only test fungus. Antagonism of *P. tinctorius* SMF towards the test fungi was also compared to that of the culture collection *P. tinctorius* strains using the same procedures. All experiments were done in triplicate.

Bioassay of antifungal activity of membrane filter-sterilized culture filtrates

Ten ml of mycelial suspension of *P. tinctorius* SMF was inoculated into a 250 ml flask containing 100 ml of optimized FMMN medium (pH 5.5–5.7). The optimized FMMN medium contained, per 1.0 liter distilled water: 3.0 g Malt Extract (Difco), 10.0 g mannitol, 0.125 g $(NH_4)_2HPO_4$, 0.05 g KH_2PO_4, 0.2 g $(NH_4)_2HC_6H_5O_7$, 0.60 g $MgSO_4 \cdot 7\,H_2O$, 0.05 g $CaCl_2$ (all from J.T. Baker), 0.05 g NaCl (EM Science, Cherry Hill, NJ), 1.0 ml FeEDTA solution, and 1.0 ml vitamin solution. Culture broth was harvested from 14-day flask cultures of *P. tinctorius* SMF grown in the optimized FMMN medium with shaking condition (120 rpm) at 25 °C. Harvested culture broth was adjusted to pH 5.5–5.7. The broth was filter-sterilized (0.45 μm pore size membrane, Nuclepore, Pleasanton, CA). This sterilized filtrate was used to prepare PDA solid medium plates where it replaced 30% of water used to prepare the medium. PDA plates not containing culture filtrate were used as a control. Colonies of each pathogen, grown on PDA plates at 25 °C in darkness for 12–24 days, were transplanted onto the PDA plates containing filter-sterilized culture filtrate and onto control plates. The plates were incubated at 25 °C in darkness. Antifungal activity of the culture filtrate was then measured as a percent of growth inhibition by comparing growth of each pathogen on both PDA + filtrate and PDA only plates.

Fermentative production of antifungal metabolites

One hundred ml of *P. tinctorius* SMF culture from a 5-day shake flask culture grown in FMMN medium was transferred to a 2.0-l glass jar fermenter (Bioflo C32: New Brunswick Scientific Co., Inc., Edison, NJ) containing 1.0 l of sterile FMMN medium. Fermentation conditions were as follows: agitation 200 rpm; pH 5.7; 25 °C; and 0.5 v/v/m aeration. Samples were taken every 6 h, and cell growth was measured by monitoring cell dry weight per ml in filtered samples. Extracellular antifungal phenolic metabolites produced by *P. tinctorius* SMF were measured by periodically recording culture medium absorbance at 316 nm (Spectronic 2000, Bausch & Lomb).

Purification and partial identification of the antifungal compounds

Active culture filtrate was prepared from harvested fermentation culture broth by removal of cell mass by filtration through Whatman no. 1 filter paper disks. The clear filtrate was then passed through an Amicon ultrafiltration cell with a PM10 membrane (M.W. cut-off 10000) (Amicon Corp., Danvers, MA) for removal of extracellular macromolecules. Bioassays (see below) were carried out at every step to track purification of the antifungal compounds. The filtered culture filtrate was freeze-dried. Different organic solvents: petroleum ether, xylene, chloroform, ethyl acetate, toluene, and methanol (all from J.T. Baker Inc., Phillipsburg, NJ) were tested for their ability to extract antifungal-active compounds from the freeze-dried culture filtrate powder. Bioassay of the solvent extracts was carried out as follows. Solvent was removed by evaporation, and then 1 ml of sterile distilled water was added to the resulting extractives to redissolve them. These solutions were placed into a hole (0.7–1.0 cm) 1.0 cm distance from *F. solani* cultures placed on PDA plates, and the free-standing water was evaporated. Antifungal activity was measured as percent of growth inhibition by comparing growth after 3 days of the pathogen on both PDA + extractives and PDA only plates.

Spectroscopic scanning was carried out to characterize the methanol extracted compounds which had antifungal activity in the bioassay. One ml of methanol was used to extract antifungal compounds from 0.2 g of freeze-dried powder. An additional 1.6 ml of methanol was added, and the solution scanned from 200 nm to 500 nm. One drop of $AlCl_3$ solution was added into the same cuvette, and the scan was repeated. $AlCl_3$ solution was prepared by dissolving 0.04 g $AlCl_3$ in 1.0 ml methanol.

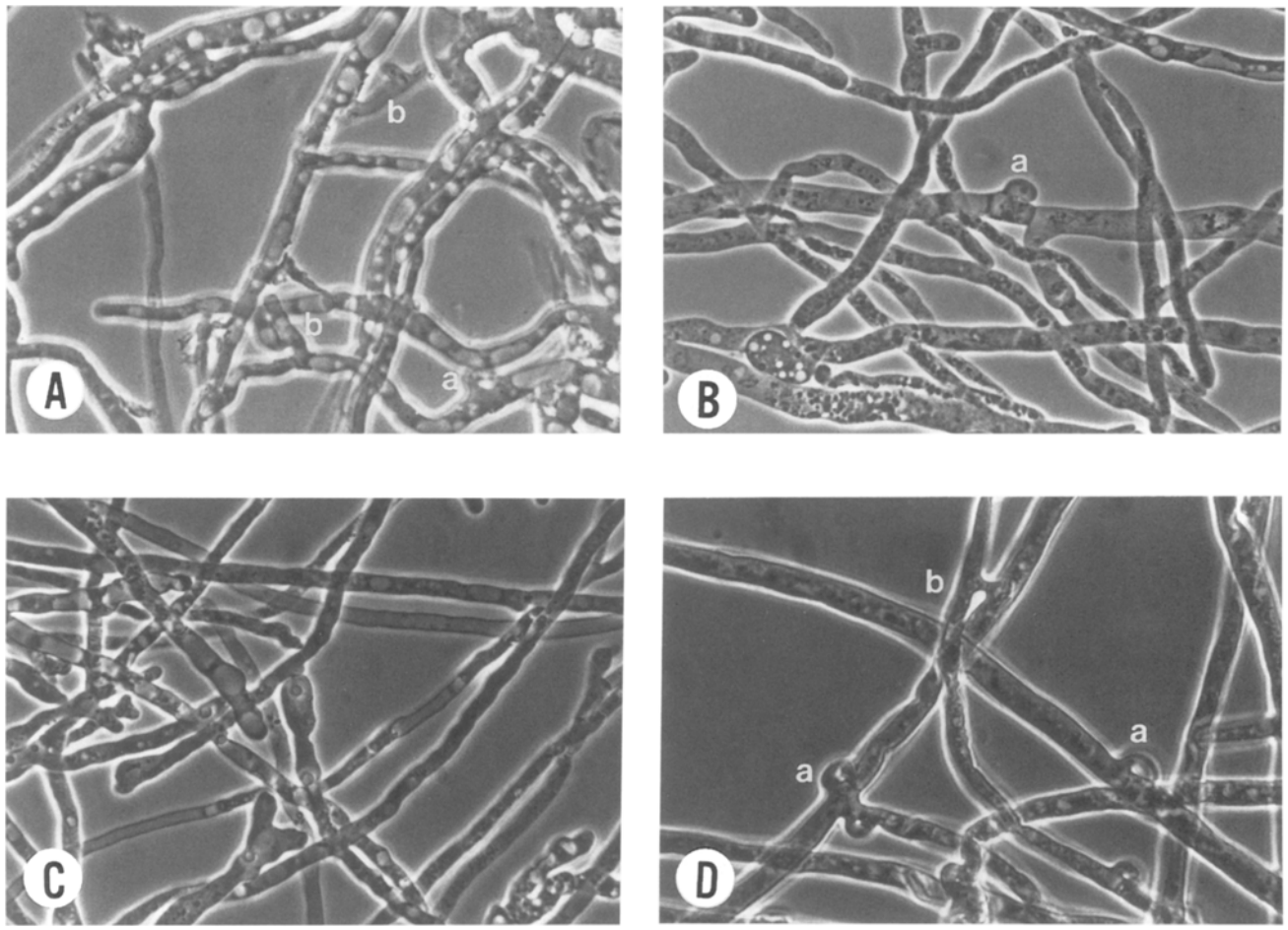

Fig. 1. Phase-contrast photomicrographs (× 400) of *P. tinctorius* SMF (A), strains 359 (B), 370 (C), and 471 (D) cultured in FMMN liquid medium. Note clamp connection (a) and specific mycelial connection (b) of both *P. tinctorius* SMF and the culture collection strains.

RESULTS

Identification of P. tinctorius SMF

Mycelial structures were investigated by microscopy after culturing *P. tinctorius* SMF and the culture collection strains in flasks containing FMMN liquid medium. Both *P. tinctorius* SMF and the culture collection strains were very similar in hyphal structure (Fig. 1). Clamp connections and specific mycelial connections were observed in all of the cultures. No contaminating bacterial cells were present.

P. tinctorius SMF formed white mycelial tips and produced diffusible dark-brown compounds. Among the culture collection strains, strains 370 and 359 formed white mycelial tips, whereas strain 471 formed brown tips. *P. tinctorius* 471 grew rapidly and also produced dark-brown compounds on PDA plates. Strain 370 did not produce the dark-brown compounds and grew very slowly on PDA plates. The growth rate of *P. tinctorius* SMF was very similar to the white type culture strain 359. *P. tinctorius* SMF grew faster than strain 370, but more slowly than strains 359 and 471.

Paired colony growth antagonism studies

During the paired colony studies, *P. tinctorius* SMF grew continuously on the PDA plates. As *P. tinctorius* SMF hyphae came close to each test fungus, the hyphal tips began to excrete brown extracellular compounds (Fig. 2). Simultaneously, growth of each fungus (*F. solani*, *V. dahliae*, *P. chrysosporium*, and *G. candidum*) on the same PDA plates were strongly inhibited as compared to control plates containing only the specific test fungus (Table 1). The growth rate of *P. tinctorius* SMF decreased, as production of the pigmented compounds increased. Clear zones of pathogen growth inhibition, indicative of the diffusion of extracellular antifungal compounds in advance of the antagonist's mycelium, were observed between *P. tinctorius* SMF and each test fungus, especially *F. solani* and *V. dahliae*. Despite the fact that the test fungi normally grew faster than *P. tinctorius* SMF on PDA plates, diffusing extracellular metabolites produced by *P. tinctorius* SMF inhibited growth of the four pathogenic fungi tested. Each test fungus, when grown alone on PDA plates, grew well, as multilayers of mycelium.

The morphologically most similar *Pisolithus* strain to *P. tinctorius* SMF, strain 359, showed extremely weak antagonism towards *F. solani* in comparison to the highly significant inhibition by *P. tinctorius* SMF (Fig. 3). Strains 471 and 370 were not at all antagonistic towards *F. solani*. In fact, mycelia of both *F. solani* and *P. chrysosporium* overgrew the mycelial margin of strains 471 and 370 in the paired colony antagonism studies.

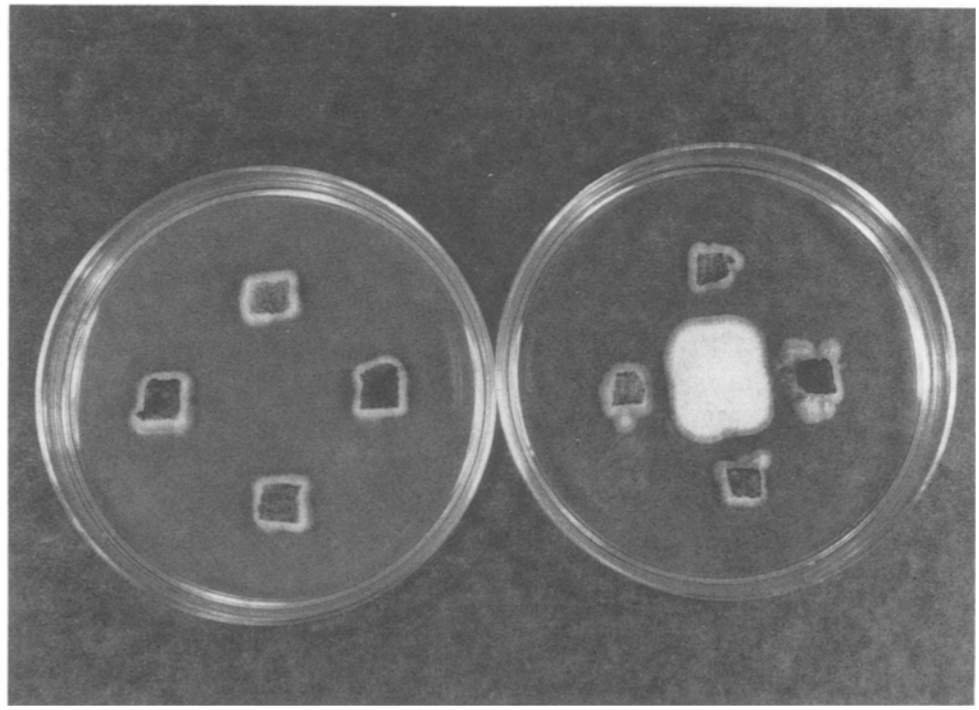

Fig. 2. Extracellular compounds production by *P. tinctorius* SMF as a defensive response to *V. dahliae* illustrating the effect of distance between *P. tinctorius* SMF and *V. dahliae*. The first (right), second (left), third (bottom), and fourth (top) shortest distance on the right side PDA plate. Left plate is the control of *V. dahliae*.

TABLE 1

Comparison of growth inhibition of pathogenic fungi by filter-sterilized culture filtrates and paired colony growth antagonism on PDA plates

Percent by which growth is reduced relative to control:				
	3 Days			5 Days
	F. solani	*P. chrysosporium*	*G. candidum*	*V. dahliae*
Filtrate	75.00 ± 2.32	62.89 ± 4.92	44.17 ± 2.79	83.10 ± 1.55
Paired colony	32.77 ± 0.94	24.08 ± 1.05	37.24 ± 0.87	64.32 ± 0.62

As a control, each pathogenic fungus was grown on PDA plates not containing filtrate or *P. tinctorius* SMF.

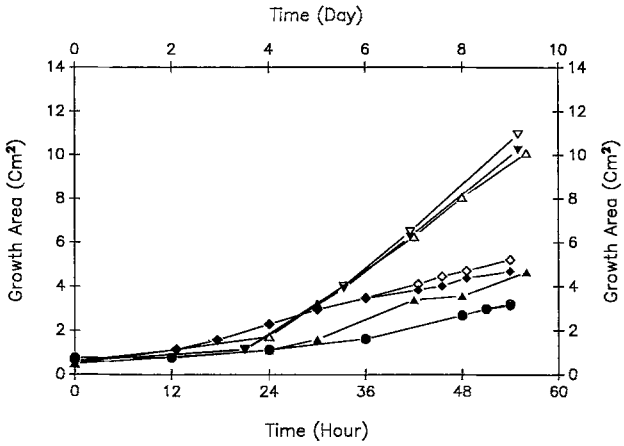

Fig. 3. Comparison of paired colony growth antagonism studies between *P. tinctorius* SMF and strain 359 against *F. solani* on PDA plates. *P. tinctorius* SMF colony area when incubated with *F. solani* (●) and *F. solani* colony area when incubated with *P. tinctorius* SMF (▲), strain 359 colony area when incubated with *F. solani* (◆) and *F. solani* colony area when incubated with strain 359 (▼). Controls: *P. tinctorius* SMF only (○) which overlaps with (●), *F. solani* only (△), strain 359 only (◇), and *F. solani* only (▽) on PDA plates.

Antifungal activity of membrane filter-sterilized culture filtrate

Filter-sterilized filtrates from *P. tinctorius* SMF cultures grown in the optimized medium possessed significant antifungal activity (Table 1). Mycelial growth of *F. solani* and *V. dahliae* was more strongly inhibited than that of *P. chrysosporium* and *G. candidum*.

Solvent extraction and spectral characterization of antifungal compounds

Methanol extracts of the filtered, freeze-dried culture filtrate powder showed strong inhibition of the mycelial growth (93 ± 2.1%) of *F. solani* in the bioassay, whereas the petroleum ether, xylene, chloroform, ethyl acetate, and toluene extracts did not.

Fig. 4a shows an ultraviolet-visible spectrum (200–500 nm) of a methanol extract which had antifungal activity. There are two peaks, with two peak absorbances of 0.854 and 0.581 at 216 nm and 248 nm, respectively. Fig. 4b shows the spectrum after addition of one drop of $AlCl_3$ solution into the same cuvette. After addition of $AlCl_3$ solution, two peak absorbances were at 216 nm and 260 nm, with absorbances of 1.823 and 1.405 respectively.

Production of phenolic-associated antifungal metabolites and cell mass in submerged culture fermentation

P. tinctorius SMF produced phenolic antifungal metabolites in much greater amounts when the fungus was cultured in submerged culture fermentation under conditions of controlled pH, aeration, and temperature than was observed in flask cultures (Fig. 5). Phenolic production was monitored by measuring culture medium absorbance at 316 nm. Growth rate was increased by a factor of approximately two as compared to the flask culture. Maximal phenolics accumulation was achieved after 108 h in the fermenter as compared to 240 h in flask cultures.

DISCUSSION

This study has demonstrated that *P. tinctorius* SMF produces extracellular compounds which inhibit growth of common species of soil-borne fungal root pathogens. The results also show that strain SMF is unique from the other *P. tinctorius* strains examined, which were not antagonistic towards the test fungi. These laboratory strains have been in culture for years and may have lost activity present at the time of their original isolation. The synthesis in vitro of antifungal compounds has been reported for over 90 species of ectomycorrhizal fungi

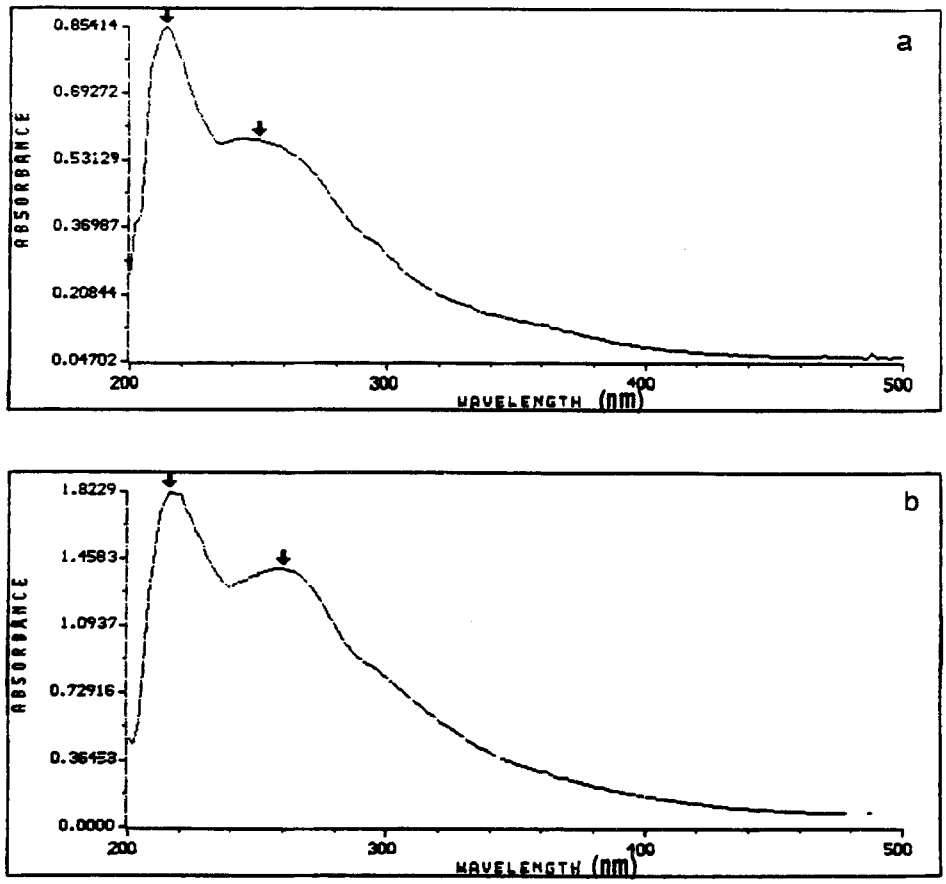

Fig. 4. Spectroscopy scans of the methanol extract from freeze dried culture filtrate powder. (a) 1.0 ml methanol extract + 1.6 ml of additional methanol. (b) 1.0 ml methanol extract + 1.6 ml of additional methanol + one drop of $AlCl_3$ solution.

[11,12,20]. However, *P. tinctorius* has not often been reported as a producer of antifungal compounds. It appears from the results presented here and by Kope and Fortin [8] that production of antifungal agents by this important ectomycorrhizal fungus is highly strain specific.

Two peak absorbances, at 216 nm and 248 nm, were present in the spectrum of antifungal metabolite-containing methanol extracts from the freeze-dried culture filtrate powder (Fig. 4). Two changes occurred upon addition of $AlCl_3$ solution. First, the 248 nm peak shifted to 260 nm.

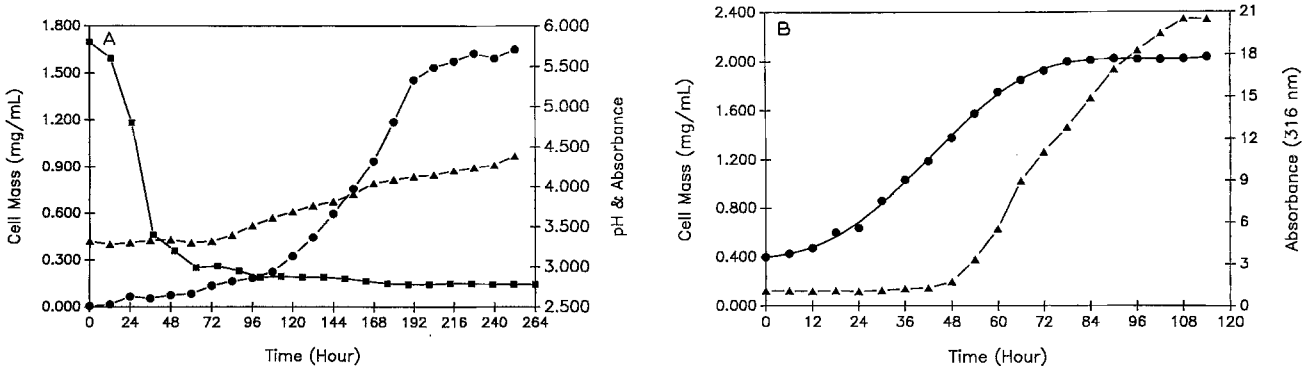

Fig. 5. Cellular growth and production of antifungal phenolic metabolites during both flask culture (A) and submerged culture fermentation (B) of *P. tinctorius* SMF. (●), cell growth (mg biomass/ml); (▼), antifungal phenolic metabolites (OD 316 nm); (■), pH.

This bathochromic shift suggests that the antifungal compounds extracted from freeze-dried culture filtrate powder are most likely phenolic compounds [5]. Also, the absorbances of the two original peaks were increased from 0.854 to 1.823 at 216 nm, and from 0.581 at 248 nm to 1.405 at 260 nm. These bathochromic and hyperchromic shifts are characteristics of phenolic compounds [5,6]. Thus, we conclude that the antifungal agent produced by strain SMF is one or more phenolic compounds. We are currently isolating and identifying the specific phenolic compounds present in these extracts.

P. tinctorius SMF grew well in submerged culture fermentation, producing substantial levels of antifungal phenolic metabolites. To our knowledge this is the first report of fermentative production of such phenolic metabolites utilizing a mycorrhizal fungus. This is an important finding because mycorrhizal fungi may be excellent, but currently untapped, sources of new antifungal compounds. Yet, little is known of their physiology and growth characteristics in submerged culture systems. Apparently, close physical association of *P. tinctorius* vegetative hyphae with those of the test fungi resulted in *P. tinctorius* synthesizing increased amounts of these phenolics on agar media (Fig. 2). This finding indicates that it may be possible to further enhance antifungal metabolite yields by *P. tinctorius* SMF in fermenter cultures.

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