Antagonism of Scytalidium Isolates against Decay Fungi

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ABSTRACT

The growth rates, celluloytic activities, production of chlamydospores, pigments, and crystals, and antagonism against four decay fungi and one blue-stain fungus of 38 European and North American isolates of three species of *Scytalidium* were compared.

Almost all isolates degraded cellulose to some degree. Three isolates of *S. aurantiacum* and 10 of 28 isolates of *S. album* killed the four decay fungi but only weakly inhibited the blue-stain fungus. Another four isolates of *S. album* killed two or three of the decay fungi. In cultures of *S. album*, yellow pigments and crystals but not chlamydospores occurred in connection with antagonism, which was characterized by secretion of a substance that caused disintegration of the hyphae of the test fungi. Some strains of *S. lignicolum* killed the decay fungi without producing significant concentrations of toxic substances, indicating a different antagonistic mechanism. After incubation with *S. album*, wood blocks contained a toxic factor which inhibited the growth of decay fungi.

The active factor was separated from the culture filtrate of one active strain of *S. album*. It was chromatographically purified and found to have a molecular weight of 527 by mass spectrograph analysis. The substance caused a 50% decrease in growth and respiration of *Fomes annosus* in artificial media at a concentration of 1 μg/ml at pH 5 but not pH 7, and stimulated the production of phenol oxidase. Pigments or crystals from cultures of active strains had no toxic effect.

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The genus *Scytalidium* was first described by Pesante in 1956 (13) and includes three species: *S. album* (Beyer & Klingström), *S. aurantiacum* (Klingström & Beyer), and *S. lignicolum* (Pesante). Since then *Scytalidium* has been isolated from blue-stained wood products including poles and pulp wood of pine and birch (2, 9, 25, 26, 27) and living trees infected by *Fomes annosus* (9). Several isolates of *S. album* are antagonistic to decay fungi both in wood and in vitro (6, 7, 8, 9, 16, 17, 18, 19, 20). The constituents of wood used as substrates for growth of *Scytalidium* are not known. Klingström & Beyer (9) found that *S. album* caused less than 2% weight loss when grown on wood. Ricard & Bollen (18) found that xylan was an excellent source of carbon in liquid culture tests.

Our objectives were (i) to determine and compare the antagonistic capacity, cellulolytic activity, and certain cultural characteristics of 38 isolates of *Scytalidium*, (ii) to determine whether antagonism against decay fungi is caused by a diffusible toxic substance or other modes of antagonism, (iii) to isolate and characterize any toxic substance(s) produced by isolates of the fungus and compare their effects with those of nonfractionated culture filtrates and mycelia of the fungi themselves, and (iv) to determine the influence of growth conditions on production of toxic substance(s).

MATERIALS AND METHODS.–The 38 isolates of *Scytalidium* tested are described in Table 1. Antagonism of these isolates was determined against: two white-rot fungi, *Fomes annosus* (Fr.) Cke. [C 40] and *Polyporus versicolor* L. ex Fr. [C 185]; two brown-rot fungi, *Lentinus lepideus* Fr. [D 97] and *Coniophora puteana* (Schum. ex Fr.) Karst. [C 54]; and one blue-stain fungus, *Leptographium lundbergii* Lagerberg & Melin [D 30]. In one test an additional white-rot fungus, *Chrysosporium* sp. [D 47] (1), was used. (Numbers in brackets refer to the Reference Collection, Department of Forest Botany, Royal College of Forestry, Stockholm).

Twenty ml of 2.5% (w/v) malt extract and 1.5% (w/v) agar were used in 9-cm petri plates for cultivation of inoculum, cross-plating tests of antagonism, and determination of the toxicity of added culture filtrates and fractions of active substances by measurement of radial growth. All cultures were incubated at 25 C. Four replicate plates were used for all antibiotic tests.

Culture filtrates of *Scytalidium* were prepared by inoculation of 50 ml of 2.5% malt extract liquid medium in 300-ml Erlenmeyer flasks with homogenized mycelium from malt extract agar cultures. These flasks were incubated at 25 C for 3 to 4 weeks.

Determination of cellulase activity on agar medium was made according to Rautela & Cowling (14).

Wood block decay tests were made according to Klingström (8).

Toxic substance(s) were purified by extraction of culture filtrates twice with ethyl ether at pH 2.5, gel

TABLE 1. Cultural characteristics and antibiotic activity of 38 isolates of *Scytalidium* on malt-extract agar medium or cellulose agar (cellulase activity tests)

Species[a]	No. of isolates	Radial growth, mm after 7 days		Isolates with chlamydospores after 9 days	Pigments formed after 9 days	Crystals formed after 28 days	Cellulase activity, mm clearing zone after 14 days	No. of fungi killed (of four) in antibiotic test
		25 C	35 C					
S. aurantiacum	3	16-20	0	0	Orange-red	None	1-4	4[b]
S. album	10	20-24	0	0	Yellow	White (brown)	4-6	4[b]
	5	20-24	0	0	Yellow	White	4-8	2-3[b]
	13	18-30	0	0	None	None	0-5	0
S. lignicolum	3	45	45	3	None	None	1-3	4[c]
	2	45	45	1	None	None	1	3[c]
	1	45	45	1	None	None	3	2[b]
	1	15	45	1	None	None	0	0

[a] Isolates with similar properties are grouped together. Antibiotic activity tested against *Fomes annosus, Polyporus versicolor, Lentinus lepideus*, and *Coniophora puteana* in cross-plating tests.

[b] Decay fungi killed in front of the advancing *Scytalidium* mycelium; agar medium yellow colored.

[c] Decay fungi rapidly overgrown and finally killed; no discoloration of the medium.

filtration with Sephadex LH-20 and ethanol:water (60:40, v/v), and preparative thin-layer chromatography (TLC) on instant TLC Silica Gel F_{254} (Merck). Active fractions were identified at all stages in the purification by bioassay with *Fomes annosus*. Laboratory columns SR 25/45 and 25/100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and a fraction collector were used for gel filtration with a pump to give a constant ascending flow rate of 0.5 ml/min. Plates for TLC were developed first in ethyl ether saturated with 0.1 M HCl. Active fractions were then rechromatographed in hexane:acetone (50:50, v/v). After elution, the active fractions were crystallized and submitted to mass spectrometry.

The direct Warburg method was used at 25 C for the respiration experiments (28). One-week-old mycelium of *Fomes annosus* grown in Norkrans' medium (12) was washed in 0.02 M phosphate buffer (pH 5 and 7, respectively) for 6 hr at 4 C, and 8-10 mg (dry wt) were suspended in 2.5 ml buffer and then added to each vessel. KOH, 0.2 ml 10% (w/v), was added to the central cup. The extract of *Scytalidium* culture filtrate or the purified substance was added to the side arm in a volume of 0.5 ml of the same buffer as in the main vessel. After O_2 uptake was measured for 60 min, the contents of the side arm was tipped into the main vessel and the respiration noted for another 90 min. Flasks without substrate and with pure buffer in the side arm were run in parallel as controls.

Laccase activity was determined manometrically using guaiacol as enzyme substrate in 0.05 M citrate-phosphate buffer at pH 4.6. The activity is given as microliters O_2 absorbed per hour and milliliters of culture filtrate of *Fomes annosus* at 25 C (24).

RESULTS.—*Cultural characteristics of Scytalidium isolates.*—The 38 isolates of *Scytalidium* were identified to species according to their growth characteristics on malt-extract agar (Table 1). Three isolates of *S. aurantiacum* from Sweden were identified by their lack of growth at 35 C, orange-red mycelium with easily discernible reddish spots, secretion of colored pigments into the medium, and formation of chlamydospores after 20-30 days which gave the culture a final black color. The three isolates originated from wood of *Betula, Pinus*, and *Picea*.

Twenty-eight isolates from Finland, Sweden, eastern Canada, and western USA were identified as *S. album* by their moderate growth at 25 C, inability to grow at 35 C, white-to-yellow mycelium, and production of black chlamydospores after 9-30 days. Crystals of various colors were found in old cultures of *S. album* only. The isolates of *S. album* originated from wood of species belonging to *Betula, Acer, Populus, Pinus, Abies*, and *Picea*.

Seven isolates from Italy, Sweden, and southern USA were identified as *S. lignicolum*. They showed rapid growth at 25 C, ability to grow at 35 C, and formation of chlamydospores after 8-9 days, resulting in a black color of the culture. The isolates of *S. lignicolum* originated from wood of *Betula, Populus, Platanus*, and *Picea* and also from *Arachis hypogaea* L. and soil.

Several isolates of all three *Scytalidium* species caused 3-5 mm of clearing in cellulose-agar substrate (Table 1), whereas *F. annosus* and *P. versicolor* caused 15-18 mm of clearing. None of 10 isolates tested caused more than 2% weight loss in spruce wood blocks. By comparison, wood-rotting fungi such as *F. annosus, P. versicolor, C. puteana*, and *L. lepideus* caused weight losses of 20-70% in wood blocks.

Antagonism of Scytalidium isolates to wood-decay fungi.—The 38 isolates and three species of *Scytalidium* differed markedly in antagonism against the wood-rotting fungi *F. annosus, P. versicolor, C. puteana*, and *L. lepideus* (Table 1). After 2 years in culture the 38 isolates showed the same relative antagonism against these same four

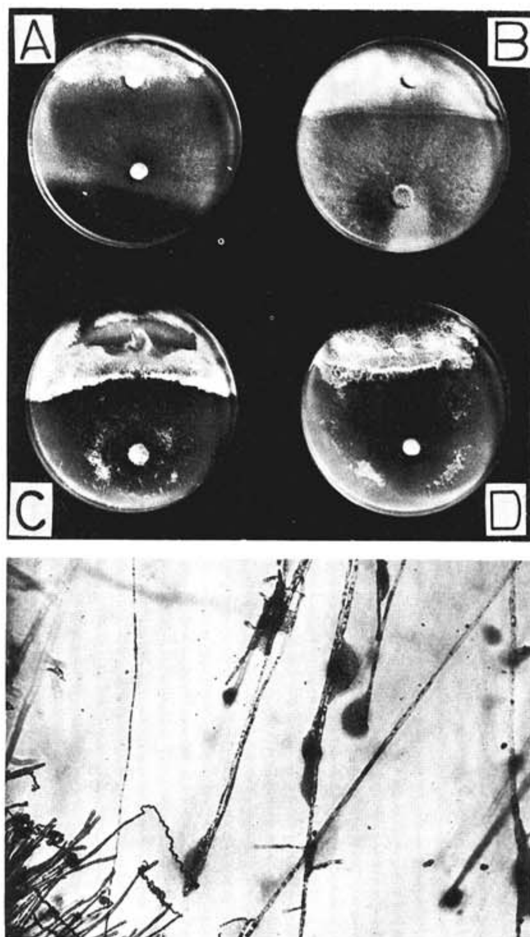


Fig. 1-2. 1) Cross-plating test with *Scytalidium album* and four decay fungi (upper part of each petri dish). A) *Fomes annosus*; B) *Polyporus versicolor*; C) *Lentinus lepideus*; D) *Coniophora puteana*. Decay fungi killed in front of the advancing *Scytalidium* mycelium. **2)** Disintegrating hyphae of *F. annosus* (upper part) in front of the advancing mycelium of *S. album*. Stained with methylene blue (ca. × 100).

fungi. The viability of the decay fungi after cross plating was tested by cultivation of mycelial pieces on Kuhlman agar (10). Decay fungi that did not grow out were considered killed by *Scytalidium*. The same test for antagonism was made with a blue-stain fungus, *Leptographium lundbergii*, but no or weak antagonism was observed.

No relationship was found between antagonism and the host or geographical origin of the isolates, but from Table 1 it can be seen that some cultural characteristics of *S. album* occur together with various degrees of antagonism. Early formation of chlamydospores is common in cultures having no antagonistic activity, whereas formation of yellow pigments and crystals was observed in cultures showing pronounced antagonism. Subsequent work, however, showed that neither the pigments nor the crystals were the source of the antibiotic activity. The three *S. aurantiacum* isolates and 10 of 27 isolates of *S. album* killed all four decay fungi. Four isolates of *S. album* killed two or three of these fungi and the remaining 13 were not antagonistic. *P. versicolor* and *L. lentinus* were more susceptible to *Scytalidium* than the two other decay fungi were.

Scytalidium lignicolum showed a different type of antagonism from that of *S. album* and *S. aurantiacum*. Hyphae of *S. lignicolum*, including the chlamydospore-forming strains, overgrew the decay fungi without inhibition. When the mixed hyphae were transferred to Kuhlman agar, the rot fungi did not grow. Isolates of *S. album* and *S. aurantiacum*, on the other hand, inhibited growth of the wood-decay fungi at a distance (Fig. 1). The peripheral hyphae of the decay fungi collapsed and disintegrated in front of the advancing mycelium of *Scytalidium* (Fig. 2). This suggests that a diffusible toxic factor is secreted by the hyphae of some isolates of *Scytalidium*. At the same time, a yellow-orange pigment appeared in the medium. When *S. album* or *S. aurantiacum* inhibited *Coniophora*, a distinct brown contact zone was formed. Sublethal concentrations of NaN_3 inhibited this darkening, indicating that a copper-containing phenol oxidase probably was secreted by *Coniophora* in response to the antagonist.

Antagonism on wood.—In excised 50-cm pieces of fresh spruce stems, two holes were bored 20-30 cm apart. A dowel with living *F. annosus* was forced into one of the holes and a dowel with active *S. album* into the other hole. After incubation for 3 months, colonization by *F. annosus* was inhibited in a yellow zone surrounding the holes inoculated with all isolates of *S. album* tested.

Sterile wood blocks were inoculated with *S. album* isolates No. 77 and No. 107. After 3 weeks the blocks were faint gray-blue and partly yellowish inside. These discolored blocks were autoclaved and then placed on agar cultures of *Poria vaporaria, L. lepideus*, and *C. puteana*. Blocks without *Scytalidium* treatment were used as controls. After incubation for 2 months, the control blocks were covered with mycelia and were partly decayed (weight loss 20-40%), whereas the blocks treated with *Scytalidium* were not covered or only sparsely covered with mycelia and showed no significant loss of weight (1-3%).

Effect of culture filtrates of Scytalidium.—*S. aurantiacum* isolate No. 61 and *S. album* isolate No. 77 were cultivated in malt-extract broth for 4 weeks at 25 C. Autoclaved filtrates from these cultures were then used in 5 and 10% concentrations to prepare malt-extract agar plates which were inoculated with mycelia of *F. annosus, L. lepideus, Chrysosporium* sp., and *L. lundbergii*. Radial growth of all four fungi was inhibited by the culture filtrates (Fig. 3). This test was repeated with several isolates of all three *Scytalidium* species using *F. annosus* and *L. lepideus* as test organisms. The results (Table 2) demonstrated that isolates of *S. aurantiacum* and *S. album* which showed antagonism in the cross-plating tests also

produced inhibitory culture filtrates. Extracts of washed and macerated mycelia of *Scytalidium* showed no significant toxic activity. This indicated that the inhibitory substance(s) was not concentrated in the mycelium.

Purification of the biologically active factor.–*Scytalidium album* isolate No. 107 was cultivated on malt-extract medium in stationary cultures for 25 days. At that time the fungicidal activity of the culture filtrate was at its maximum and the pH was 3.0-3.5. Samples of the culture filtrate were adjusted to pH values between 2.5 and 8.0 and then extracted with ethyl ether. The active factor could only be extracted at acidic pH values. At pH 2.5 the toxin was almost quantitatively transferred to the ether phase by two extractions. The active fraction was separated from yellow pigments by gel filtration of the ether extract after it was dissolved in ethanol. The pigments were collected in two fractions with molecular weights of approximately 400 and 500, respectively; they had no antibiotic activity. By TLC in two solvent systems, a single active fraction was obtained with white-yellow fluorescence at 366 nm and an extinction at 254 nm. This fraction was eluted from the silica gel with methanol or acidic ethyl ether. Mass spectrometry of the product gave a well-defined peak at m/e 527, which was considered to be a molecular peak. The substance was heat-stable to 120 C and slightly soluble in solvents such as methanol, ethanol, acetone, ethyl methyl ketone, ethyl ether, ethyl acetate, and hexane.

About 1 μg/ml of this pure substance inhibited mycelial growth of *F. annosus* about 50% both in agar and in liquid culture tests (Fig. 4, 6). This effect is comparable to that caused by cycloheximide, an antibiotic previously studied in connection with *F. annosus* (5). Growth-inhibiting ether extracts could also be obtained from wood pieces previously inoculated with *Scytalidium*.

Endogenous respiration of *Fomes* mycelia at pH 5 was strongly decreased by growth-inhibiting concentrations of filtrates and purified fractions from *Scytalidium* cultures (Fig. 5). At pH 7 no effect on respiration was obtained. When pH was readjusted to 5, the effect was restored. Parallel tests clearly showed that the same substance inhibited growth and respiration. The importance of pH has been verified by growth tests; no inhibition was obtained at pH 7.0.

Stimulation of the activity of phenol oxidase of laccase type was obtained when sterilized culture filtrate or purified growth-inhibiting substance from *S. album* cultures was added to cultures of *F. annosus* (Fig. 6). The same effect was obtained with many fungitoxic substances; e.g. lignin phenols (vanilline), stilbenes (pinosylvin), simple phenols (catechol), or uncoupling agents (2,4-dinitrophenol).

Effect of various culture conditions on toxin production.–Preliminary tests showed that shake cultures produced significantly smaller amounts of antibiotic substances than stationary ones did. Reduction of the oxygen pressure in flasks of

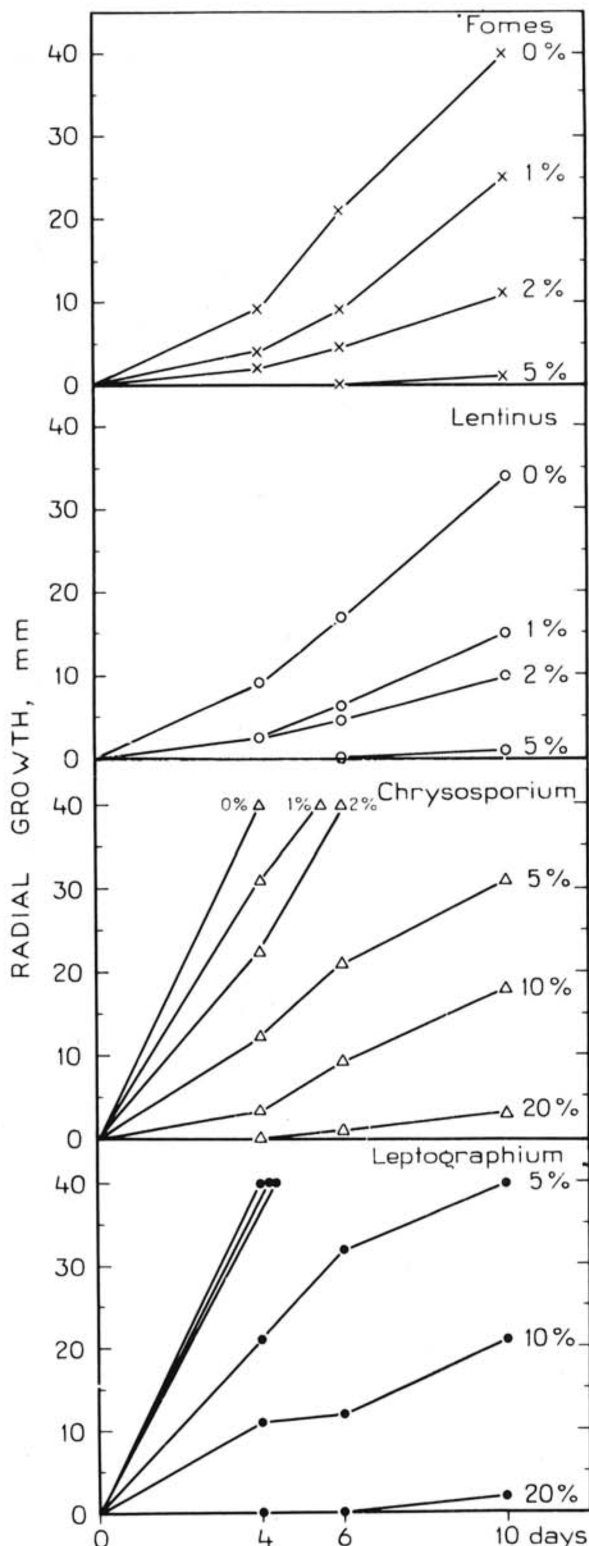


Fig. 3. Effect of various concentrations of 3-week-old culture filtrate from *Scytalidium album* (isolate no. 77) added to malt-extract agar on radial growth of *Fomes annosus*, *Lentinus lepideus*, *Chrysosporium* sp., and *Leptographium lundbergii*.

TABLE 2. Percentage inhibition of radial growth of *Lentinus lepideus* and *Fomes annosus* on malt-extract agar caused by addition of 5 and 10% sterilized culture filtrate from 4-week-old cultures of *Scytalidium*

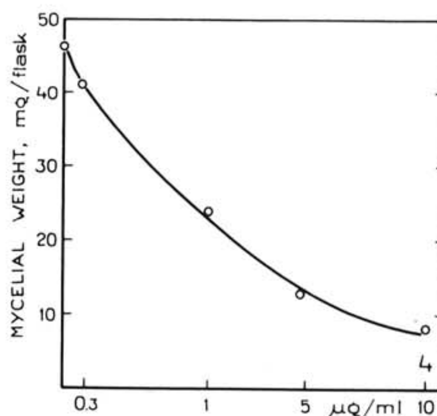
Culture filtrate from	Inhibition[a] by 5% culture filtrate		Inhibition[a] by 10% culture filtrate	
	Lentinus	*Fomes*	*Lentinus*	*Fomes*
	%	%	%	%
S. aurantiacum				
Strain no. 61	64	60	93	78
105	10	21	25	45
S. lignicolum				
Strain no. 7	18	11	36	10
59	25	0	32	22
89	25	11	43	22
S. album				
Strain no. 26		15		22
62		13		50
75		0		0
76	46	69	71	98
77	96	100	100	100
80		13		50
99		27		53
103		0		0
107	75	80	100	100
108		6		24
112		69		83
114	54	50	93	98
117		5		50

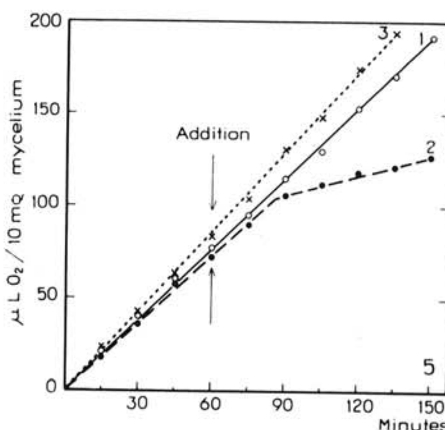
[a] Incubation time was 8 days. Mean of four petri dishes.

stationary cultures with carbon dioxide or nitrogen did not increase the production.

When testing the effect of various carbon sources on the mycelial growth of a strain of *S. album*, Ricard & Bollen (18) reported a remarkably high yield when xylan was added to a diluted malt-extract medium. We repeated this experiment in connection with studies of the growth-inhibiting properties of the culture filtrates. None of the carbohydrates added stimulated production of mycelia or antibiotic more than malt extract. Xylans from various sources stimulated growth to a limited extent in malt-extract medium and, depending on the origin of xylan, some stimulation of production of antibiotics was obtained. A constant effect of xylan in this respect has not been elucidated; however, *F. annosus* did not produce substances that stimulated antibiotic production. No significant differences as to antibiotic production were obtained in liquid cultures whether the inoculum was taken from single cultures of *Scytalidium*, from *Scytalidium* in cross-plating tests with *F. annosus*, or from wood substrates.

DISCUSSION.—The three species of *Scytalidium* used can be regarded as blue-staining Fungi Imperfecti. They have a very wide geographical distribution and are relatively common in a number of wood substrata and even in poles treated with creosote oil. Substantial variation in various





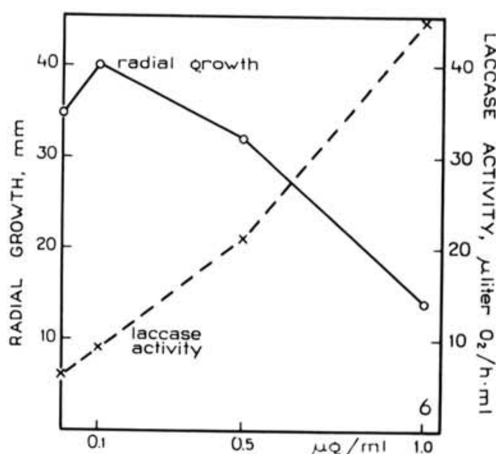


Fig. 4-6. Effects of purified antibiotic from *Scytalidium album* (isolate no. 107). **4)** On mycelial dry weight production of *Fomes annosus* in liquid culture. Incubation time of *F. annosus* was 14 days. **5)** On endogenous respiration of *F. annosus.* 1) Control, pH 5; 2) antibiotic added, pH 5; 3) antibiotic added, pH 7. The arrows indicate the time of addition of antibiotic. **6)** On radial growth and laccase activity in agar culture of *F. annosus*. Incubation time of *F. annosus* was 8 days.

physiological properties was observed among the isolates tested.

Yellow pigments usually appear in agar and liquid cultures of isolates that are antagonistic to decay fungi. Thus, formation of pigments is not unique to the FY strain (*S. album* isolate No. 77 in our tests) as asserted by Ricard et al. (20). By both gel filtration and TLC we have shown that the yellow pigment can be separated into at least two fractions. This also contrasts with the single component found by Ricard.

We have shown that the formation of easily discernible crystals is common in agar media with *S. album*. The strains of *S. album* studied by Ricard & Bollen (18) provided the first example of crystal formation. They considered that these crystals contained the antibiotic substance(s) (17, 18, 20). However, such crystals obtained in our tests showed no antibiotic activity. The crystals were formed in agar cultures of isolates from very different geographical areas. The chemical composition of these crystals is not yet defined (17). On the other hand, we found that the ability of *S. album* isolates to kill decay fungi in agar and liquid media is due to their production of a slightly water-soluble toxic substance. This substance is also produced in wood substrates and is active, even though the fungus is killed by autoclaving.

The inhibition zone which is produced in cross-plating tests on solid media and the secretion of the antibiotic substance(s) by young cultures in liquid media suggest that the antagonistic factor is a product of actively growing mycelium and not of autolysis.

The total amount of biological activity of the purified substance was generally less than one-third of that of the corresponding culture filtrate. This may be caused by losses during the TLC separation, alteration of the solubility of the substance during purification, or loss of a synergistic or other factor(s) present in the culture filtrate.

The difference in the physiological effects of the substance obtained at pH 7 and at pH 5 might be a question of permeability through the cell membranes. At the higher pH value the substance might occur as an anion, which does not penetrate the cell membrane as easily as the more electrically neutral and lipophilic form of the substance existing at a lower pH. This is in good agreement with the solubility of the substance in ethyl ether at the two pH values.

Ricard & Laird (19) suggested that competition with wood-destroying fungi is required for maintaining the capacity for effective biosynthesis of antagonistic substances by *S. album* isolate No. 77. Our isolates were equally antagonistic to *F. annosus* and three other decay fungi after 2 years in malt-agar cultures. We do not know why shake cultures are inferior to stationary ones in production of antibiotic substances. On the basis of studies with reduced oxygen pressure, this is not a simple question of the concentration of oxygen in the culture medium.

This investigation has shown that several isolates of *Scytalidium* exhibit an antibiotic effect both in vitro and in wood substrates. It has been suggested that *Scytalidium* could be used for biological control of decay fungi such as *Poria carbonica* in utility poles or pathogens like *F. annosus* in spruce trees (16, 18, 19). Our results from cultivation of *S. album* and *S. aurantiacum* on wood blocks and tree stems show that antibiotic products are deposited in the wood without significant loss of weight of wood substance (strength loss or other criteria were not studied). This is in accordance with the results obtained by Klingström & Beyer (9) with one isolate of *S. album*. Preliminary field tests by us have shown that isolates of *S. album* which exhibited antagonistic activity in cross-plating tests were also capable of eliminating or inhibiting the spread of decay fungi when inoculated into spruces attacked by *F. annosus*.

Antagonistic effects between *F. annosus* and other fungi or bacteria have been mentioned by Rishbeth (21, 22, 23), Rennerfelt (15), Björkman (3), Gundersen (4), and Nissen (11). Hyppel (7) summarized a number of experiments dealing with mycorrhiza fungi as antagonists of *F. annosus*.

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