Antifungal Spectra of Actinomycetes Isolated from Tobacco¹

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Five species (28 strains) of actinomycetes isolated from tobacco were tested for antagonism against 12 species of storage and field fungi associated with tobacco. Two strains of *Streptomyces albus* were antagonistic against all test fungi. The actinomycetes grew more rapidly, produced more pigment, and had more pronounced antibiotic activity when grown at 36 C than at 28 C. Krasilnikov's synthetic medium, SMK-1, supported the greatest antifungal activity. More of the actinomycetes were antagonistic against more test fungi when grown for 20 days rather than 10 days.

Previous investigations demonstrated that many different microorganisms are associated with tobacco leaves during the growing season, at harvest, and in storage (4, 8, 9; Lukic and Lucas, *submitted for publication*). Dried tobacco that becomes wet or is stored under humid conditions is susceptible to mold or damage (10). The principal fungi that grow in moist stored tobacco are species of *Aspergillus* and *Penicillium* (9). Tesic and Lukic (5) showed that *Actinomyces coeruleus* Baldacci isolated from soil was strongly antagonistic to molds commonly associated with stored tobacco, and preliminary experiments indicated that moldiness could be prevented by spraying stored tobacco with culture filtrates of this organism. They suggested that stored tobacco might be sprayed with antibiotics to prevent fungal decay.

The purpose of this investigation was to determine the antagonistic spectra of the 28 strains of actinomycetes isolated by Lukic and Lucas (*submitted for publication*) against 9 species of storage fungi and 3 species of field fungi isolated from flue-cured tobacco.

MATERIALS AND METHODS

Five species of streptomycetes (28 strains), as described by Lukic et al. (*submitted for publication*), were tested for antagonism to 12 species of storage and field fungi isolated from flue-cured tobacco (9). Test fungi were *Aspergillus niger* (Van Tiegham), *A. ochraceus* Wilhelm, *A. flavus* Link, *A. tamarii* Kita, *A. candidus* Link, *A. ruber* (K. S. & B.) Thom &

Church, *A. amstelodami* (Margin) Thom & Church, *A. repens* DeBary, *Penicillium cyclopium* Westling, *Alternaria alternata* (Fr.) Keissl., *Cladosporium cladosporioides* (Fres.) comb. n., and *Epicoccum nigrum* Link.

Streptomyces albus (Rossi Doria) Waksman & Henrici, *S. diastaticus* (Krausky) Waksman & Henrici, *S. flaveolus* (Waksman) Waksman & Henrici, *S. griseus* (Krausky) Waksman & Henrici, and *S. venezuelae* Ehrlich et al., were grown on five media to determine the effects of substrate on the production of antagonistic compounds:

(i) Soybean meal-yeast extract-glucose (SYG) agar was based on the medium A-4h of Warren et al. (7 and T. G. Pridham, *personal communication*) and contained: soybean meal, 15 g; yeast extract, 1 g; Difco D-glucose, 15 g; glycerol, 2.5 g; NaCl, 5 g; CaCO₃, 1 g; Difco agar, 15 g; distilled water, 1,000 ml. The pH was not adjusted.

(ii) Synthetic agar 1 (SMK-1) Krasilnikov (3) consisted of K₂HPO₄, 3 g; MgCO₃, 0.3 g; NaCl, 0.2 g; KNO₃, 1 g; FeSO₄, 0.001 g; CaCO₃, 0.5 g; glucose, 20 g; Difco agar, 20 g; tap water, 1,000 ml; pH 7.0 to 7.2.

(iii) Synthetic agar 4 (SMK-4) Krasilnikov (3) consisted of: KNO₃, 0.1 g; MgSO₄, 0.05 g; KCl, 0.07 g; KH₂PO₄, 0.1 g; Ca(NO₃)₂, 0.25 g; glucose, 20 g; Difco agar, 20 g; tap water, 1,000 ml; pH 7.0 to 7.2.

(iv) Soybean meal agar (CSM) Ciganov (2) contained: soybean meal, 10 g; glucose, 10 g; NaCl, 5 g; CaCO₃, 1 g; Difco agar, 20 g; tap water, 1,000 ml; pH 6.8.

(v) Beef-extract agar (WM) Waksman (6) contained: peptone, 10 g; beef extract, 5 g; glucose, 20 g; NaCl, 5 g; Difco agar, 20 g; tap water, 1,000 ml. The pH was not adjusted.

Egorov's (1) method for determining growth inhibitors produced by actinomycetes was used, with the following adaptation. A 30-ml amount of medium was

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FIG. 1. Instrument used to simultaneously inoculate six test fungi. The instrument is dipped into the reservoirs in the acrylic plastic container (right), each containing spore suspensions of a given test fungus, then lightly pressed to the medium (left) on which an agar plug containing the actinomycete inoculum had been placed 24 hr earlier.

TABLE 1. Number of strains of actinomycetes on different media exhibiting activity against fungi commonly associated with stored tobacco

Media ^a	Fungi tested ^b												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
	After 10 days of growth												
SMK-1	24	17	6	5	7	13	16	19	8	6	9	10	140
WM	20	4	1		2	10	16	15	3	5	6	4	86
SMK-4	11	6	7	6	7		6		10	8	1		62
CSM	13	2	1	2			6	7	5	5	7		42
SYG	11						2	11	3	2	1		30
	After 20 days of growth												
SMK-1	26	26	9	7	5	16	14	26	9	6	12	17	173
WM	22	8	3		3	8	15	17	3	1	4	5	89
SMK-4	13	5	8	8	7	1	6	1	11	7	3		70
CSM	12		1		7	7	13	16	1	4	8	2	71
SYG	18	1	1		3	11	8	9	3	3	3	8	68

^a Media: SMK-1, Krasilnikov's synthetic agar I; SYG, soybean meal-yeast extract-glucose agar; SMK-4, Krasilnikov's synthetic agar IV; CSM, Ciganov soybean meal agar; WM, Waksman's beef-extract agar.

^b 1, *Aspergillus niger*; 2, *A. ochraceus*; 3, *A. flavus*; 4, *A. tamarii*; 5, *A. candidus*; 6, *A. ruber*; 7, *A. stielodami*; 8, *A. repens*; 9, *Penicillium cyclopium*; 10, *Alternaria alternata*; 11, *Cladosporium cladosporoides*; 12, *Epicoccum nigrum*.

poured into a 9-mm diameter petri dish, inoculated with one strain of actinomycete, and incubated for either 10 or 20 days at 28 or 36 C. A 2.5-cm diameter plug of the medium and the actinomycete was transferred aseptically to the center of a petri dish containing 15 ml of Czapek's medium. These petri dishes were incubated in the dark at room temperature for 24 hr and then inoculated simultaneously with six test fungi. The specially made inoculating instrument (Fig. 1) is equipped with six bent needles attached to a plastic disc. Individual needles were dipped simultaneously into the six individual reservoirs, each containing a spore suspension of a given test fungus, and then

lightly pressed to the Czapek's medium. Care was taken not to disturb the plug containing the actinomycete inoculum in the center of the dish. After inoculation, the petri plates were incubated at 28 C; growth inhibition zones were measured after 3 and 6 days.

RESULTS

In preliminary tests when actinomycetes were incubated at 28 C the colonies grew slowly, produced little pigment, and showed only slight activity against the test fungi. However, when incubated at 36 C, the colonies grew more rapidly,

produced more pigment, and had pronounced antibiotic activity. Of the five substrates tested, Krasilnikov's synthetic medium, SMK-1, supported the greatest antifungal activity. On this medium, 27 strains exhibited activity against *Aspergillus niger*, *A. ochraceus*, and *A. repens*; 22 to *A. ruber*; 20 against *A. amstelodami* and *Epicothium nigrum*; and 16 to *Cladosporium cladosporioides*. Only nine strains were antagonistic to *Penicillium cyclopium* and seven strains to *A. tamarii* and *Alternaria alternata*. Two strains, no. 7 and 8, of *Streptomyces albus* showed activity against all 12 test fungi. Generally, *A. niger* and *A. repens* were more susceptible to the activity than the other fungi tested. *A. tamarii* was the least susceptible.

Inhibition zones on SMK-1 ranged from 0 to 20 mm and averaged 5.9 mm. On SYG, SMK-4, CSM, and WM, inhibition zones usually were less than 20 mm and averaged only 1.9, 1.5, 1.7, and 2.5 mm, respectively.

Antifungal activity was directly related to time of incubation (Table 1). More test fungi were inhibited by more strains of actinomycetes after 20 days than after 10 days of incubation.

DISCUSSION

Substrate composition influences antibiotic production by actinomycetes (2, 3, 5, 6). This is confirmed by our tests wherein strains of actinomycetes grown in SMK-1 showed more activity against test fungi on this medium than on the other four media.

Temperature also affects antibiotic production (2, 3, 5, 6). However, the increased antifungal activity at 36 C seems somewhat at variance with usual observations about optimal temperature for production of antibiotics by actinomycetes (Pridham, *personal communication*). Additional studies on the effects of temperature, and perhaps of

other factors, on the production of antifungal substances might be fruitful.

Confirmation of the fact that actinomycetes produce compounds inhibitory to mold growth gives further credence to the idea, as proposed by Tesic and Lukic (5), that antibiotics might be used to protect tobacco or other stored agricultural products from damage by fungi, insofar as these methods are practical and economical.

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