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Mouse Toxicity of Fungi of Tobacco[1]

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A bioassay for fungal toxins based on the intraperitoneal injection of test materials into mice was used to screen 976 cultures isolated from tobacco and grown in a high-protein baby cereal and also to determine whether samples of tobacco damaged by fungi are more toxic than samples of apparently sound tobacco. Of 236 fungal isolates from noncured tobacco, 79% were lethal when homogenized cultures of these isolates were tested. Forty-nine per cent of 740 fungi isolated from cured tobacco were lethal. Of the genera from which 30 or more isolates were tested, *Epicoccum*, *Alternaria*, and *Penicillium* had the highest percentage of toxic isolates from noncured tobacco, whereas *Epicoccum*, *Aspergillus*, and *Alternaria* had the highest percentage from cured tobacco. Samples of tobacco naturally infected with brown spot, caused by *Alternaria tenuis*, did not have a significantly different LD_{50} value after 48 hr than comparable disease-free samples. However, animals which died from doses near the LD_{50} dose of tobacco infected with *Alternaria* generally died in 24 to 48 hr with signs associated with a depressant rather than a stimulant, such as nicotine, which caused death in 15 to 30 min. These signs were duplicated by injecting homogenized pure cultures of *Alternaria*. These studies, although inconclusive with regard to the effects of fungal contaminants on the quality or usability of tobacco, have developed evidence that suggests the advisability of a study on smoke or smoke condensates from moldy and nonmoldy tobacco.

Flue-cured tobacco, *Nicotiana tabacum* L., is one of the principal ingredients of cigarettes and other manufactured tobacco products. The harvested green leaves are known to harbor a wide variety of microorganisms most of which are nonpathogenic to the growing plant (8). The cured leaf, especially after handling, marketing, and storage, also harbors an extensive fungal flora (8; J. Forgacs and R. Barrack, Bacteriol. Proc., p. 1-2, 1968) which differs somewhat from that found on the green leaves. Cause and effect relationships between these fungi and the quality or usability of tobacco have not been established.

Recently, attention has been focused upon the fungal contaminants of tobacco because fungi are known to affect the quality of agricultural commodities, particularly feed and feed ingredients. Although the toxicity of moldy feeds to warm-blooded animals has been known for a long time (9), few investigations appear to have been conducted on the toxicity of the fungal flora of tobacco. Forgacs and Carll (2) reported that spores of an unidentified species of *Alternaria* were found in cigarette tobacco and that when mice inhaled smoke generated from cultures of *Alternaria* and *Aspergillus niger* Van Tiegham grown on timothy hay, the animals developed emphysema. Smoke from uninoculated hay did not cause any discernible tissue response except chronic inflammation. It was not clear from their report if the fungi were originally isolated from tobacco, nor have there been other reports of attempts to extend these findings from hay to tobacco. Doupnik and Sobers (1) reported that corn inoculated with *Alternaria longipes* (Ellis and Everhart) Mason isolated from tobacco was toxic when fed to chicks.

Theoretically, the presence of fungi in tobacco may be inconsequential, beneficial, or harmful. If they improve the flavor and aroma of the tobacco and are not noxious, they may improve smoking quality and thus be desirable. On the other hand, if they impart an unpleasing flavor or aroma or produce a toxic substance or precursor carried in tobacco smoke, they would be undesirable. This investigation was begun to clarify some of the diverse facets of the effect of fungi in tobacco. Because smoke tests are so expensive in terms of capital, time, and labor that a large scale screening program is prohibited, it was decided

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to do the initial screening by using intraperitoneal (ip) injections of the test materials. In comparison, this route is an easy, rapid, and sensitive way of demonstrating toxicity. The present communication reports the results of ip injections of samples from 976 fungi isolated from tobacco and tobacco infected with *Alternaria tenuis* Nees.

MATERIALS AND METHODS

Toxicity tests. Female Swiss albino DUB/ICR mice (18 to 24 g, from Dublin Animal Laboratory, Dublin, Va.) were used for the toxicity studies. Lethality assays on whole cultures were done by ip injection at dosage levels of 0.030, 0.010, and 0.003 ml per g of mouse body weight. Injection of the culture media and diluent at these dosage levels was without effect. The LD_{50} values of tobacco samples were determined by the method of Miller and Tainter (5) by using 5 dosage levels and 6, 8, or 10 animals per dose depending upon the amount of sample available. The mice were observed for 48 hr after injection, and the number of deaths by this time was used for calculating the LD_{50} value. Postmortem examination, including histopathology on 10 bodies selected at random, revealed no signs of foreign body reactions or infectious processes. About 600 survivors from these experiments were kept for 2 months, and 12 of them developed palpable granulomas, whereas 2 mice developed a fungal infection of the organs in the peritoneal cavity.

Source of fungi. A previous paper (8) reported on the fungi and the frequency of their isolation from tobacco before and after flue-curing. The fungi tested here for toxicity were selected at random for the laboratory collection of all of these isolates, and thus they presumably represent the naturally occurring fungal profile.

Preparation of cultures for injection. Cultures tested for toxicity were grown in 100 ml of a 6% suspension of High Protein Cereal for Baby (Gerber Products Co., Freemont, Mich.) in 500-ml Erlenmeyer flasks. The inoculated flasks were incubated for 7 days at 25 C on a rotary shaker at 210 rev/min with a 1-inch (2.54 cm) radius of rotation. The entire contents of the flask were comminuted in a food blendor for 30 sec and further homogenized in a tissue grinder. Dilutions of the homogenized cultures were made in sterol diluent (10) to yield stable dispersions. The homogenized cultures were stored at 4 C for up to 6 hr before injection. Longer storage was at −70 C. Injections of sterile medium were found to be nontoxic.

Source of tobacco. Samples of tobacco were obtained from experimental field plots at three locations in North Carolina (7). The plants in these plots exhibited a severe natural infection of *A. tenuis* Nees, the causal agent of brown spot, and some had been sprayed with maneb (manganese ethylene-bis-dithiocarbamate) to control the disease. Since the LD_{50} dose of maneb is 6,750 mg/kg (6) or about 10 times the LD_{50} of the experimental material, its possible presence was not considered a significant factor. Twenty leaves were selected at random from the top one-third of plants growing in both the sprayed and nonsprayed plots. Toxicity determinations were made on disease tissue excised from leaves of the nonsprayed plants and similarly from portions of apparently healthy leaves from sprayed plants.

Preparation of tobacco for injection. Tobacco samples were dried in a vacuum desiccator over con-

TABLE 1. *Toxicity of homogenized cultures of fungi isolated from noncured tobacco as determined by ip injection of mice*

Organism	No. of isolates tested	Percentage of toxic isolates	No. of isolates toxic at indicated dosage[a]		
			0.030	0.010	0.003
Epicoccum	104	98.0	59	40	3
Alternaria	38	78.9	19	5	6
Penicillium	37	78.3	26	3	0
Cladosporium	15	46.6	5	2	0
Trichoderma	10	70.0	4	3	0
Chaetomium	9	44.4	4	0	0
Fusarium	6	—[b]	3	0	0
Nigrospora	6	—	2	0	0
Helminthosporium	3	—	1	0	0
Pestalotia	3	—	0	0	0
Aspergillus amstelodami	1	—	0	0	0
A. versicolor	1	—	1	0	0
Dendrophoma	1	—	1	0	0
Phoma	1	—	0	0	0
Rhizoctonia	1	—	0	0	0
Totals	236		125	53	9

[a] Minimal lethal dose, in milliliters per gram, that killed two of two mice.
[b] The presentation of per cents is omitted because they are based on too few items.

centrated sulfuric acid and ground to a fine powder with a mortar and pestle. Weighed amounts of the powder were homogenized in sterol diluent before injection into mice.

Statistical evaluation of toxicity data. The toxicity data from the injection of tobacco were subjected to an analysis of variance. The significance of the difference between the means of infected and disease-free tobacco and between the source means was determined by calculating the least significant difference.

RESULTS

The toxicity of 236 fungal isolates from noncured tobacco is shown in Table 1. Of the isolates from this tobacco source, 79% were toxic to mice at doses of 0.030 ml/g, which was the highest dosage tried. Fungi from the genera *Epicoccum*, *Alternaria*, and *Penicillium* accounted for 76% of the isolates tested. Of the isolates from these genera, 90% were toxic. These genera also included the nine isolates which were lethal at the highest dilution tried (0.003 ml/g of mouse body weight). The other genera from which nine or more isolates were tested were *Cladosporium*, *Trichoderma*, and *Chaetomium*. Of the 34 isolates from these genera, 53% were lethal.

Forty-nine per cent of 740 fungal isolates from cured tobacco were toxic to mice (Table 2). Although three times as many isolates were tested from cured tobacco as from noncured tobacco (740 versus 236), a smaller percentage of the isolates from cured tobacco were toxic. In addition, proportionally fewer isolates from cured tobacco were toxic at the high dilutions (low dosages) than were the isolates from noncured tobacco. Of the 212 isolates of *Alternaria* tested, 60.4% were toxic as were 81.8% of 33 isolates of *Epicoccum* tested. Thirty-eight isolates of *Botrytis* were tested and 55.2% were toxic. Of the 276 isolates of *Chaetomium*, *Nigrospora*, and *Penicillium*, 37.3% were toxic, whereas 54.5% of the 88 isolates of seven species of *Aspergillus* were toxic. There was a greater percentage of toxic isolates of *A. niger*, the *Aspergillus* species most frequently tested, than of the other *Aspergillus* species. Isolates from 18 other genera were tested, and the incidence of toxicity for individual genera ranged from 0 to 100% and averaged 43.5% for the 131 isolates.

After establishing that some of the fungi isolated from tobacco were toxic, it was desirable to determine whether tobacco damaged by fungi was more toxic than nondamaged tobacco. A test of fungal-induced toxicity in tobacco was provided by comparing the toxicity in mice of preparations of tobacco leaves naturally infected with *A. tenuis* and of fungicide-treated leaf tissue apparently free from brown spot (Table 3). The tobacco preparations made from cured leaves infected by *A. tenuis* generally were not significantly more toxic than preparations from leaves free from brown-spot disease. There was a significant difference in toxicity of tobacco from different sources. However, indications of a difference between healthy and infected leaves were noted in the physiological signs and time of death of the injected mice. The mice which died from an injection of a preparation from apparently sound leaves died within 15 to 20 min, and the physiological signs displayed were identical, as expected, to those elicited by injections of pure nicotine. Vasodilation and mydriasis preceded persistent clonic convulsions which led to vaso-

TABLE 2. *Toxicity of homogenized cultures of fungi isolated from cured tobacco as determined by ip injections of mice*

Organism	No. of isolates tested	Percentage of toxic isolates	No. of isolates toxic at indicated dosage[a]		
			0.030	0.010	0.003
Alternaria	212	60.4	84	40	4
Chaetomium	134	36.5	39	5	5
Nigrospora	99	38.3	37	1	0
Penicillium	43	37.2	16	0	0
Botrytis	38	55.2	20	0	1
Epicoccum	33	81.8	15	9	3
Aspergillus niger	30	80.0	24	0	0
Cladosporium	29	48.2	11	3	0
Stemphyllium	21	19.0	4	0	0
Aspergillus ruber	17	23.5	4	0	0
A. amstelodami	13	46.1	6	0	0
A. versicolor	12	33.3	4	0	0
A. flavus	10	60.0	6	0	0
Curvularia	10	10.0	0	1	0
Helminthosporium	8	—[b]	2	0	0
A. ochraceus	5	—	3	0	0
Candida	3	—	3	0	0
Fusarium	3	—	3	0	0
Sordaria	3	—	0	0	0
Trichoderma	3	—	1	0	0
Cephalosporium	2	—	1	0	0
Dendrophoma	2	—	1	0	0
Phoma	2	—	1	0	0
Spegazzinia	2	—	2	0	0
Aspergillus repens	1	—	1	0	0
Cylyndrocephalum	1	—	0	0	0
Pestalotia	1	—	1	0	0
Pullularia	1	—	1	0	0
Rhizoctonia	1	—	0	0	0
Stigmella	1	—	1	0	0
Totals	740		291	59	13

[a] Minimal lethal dose, in milliliters per gram, that killed two of two mice.

[b] The presentation of per cents is omitted because they are based on too few items.

TABLE 3. *Toxicity to mice of ip injections of A. tenuis-infected and brown spot-free cured tobacco*

Source	Tobacco free from brown spot	Tobacco infected with *A. tenuis*	Source means
A[a]	340[b]	240	
	620	370	
	620	440	
Means	527	350	438[c]
B	155	103	
	162	150	
	190	150	
Means	169	134	151[c]
C	250	255	
	300	380	
	420	—[d]	
Means	323	317	320[c]
Overall means	340[e]	261[e]	

[a] The samples from each source were taken randomly, and no comparison is intended among the individual samples which are ranked arbitrarily by increasing LD_{50} values.

[b] The LD_{50} value in milligrams per kilogram.

[c] Means with this superscript are significantly different from each other ($P < 0.05$).

[d] The number 317 was added into the mean to restore symmetry for the analysis of variance. A degree of freedom was subtracted to compensate.

[e] Means with this superscript do not differ significantly.

constriction, cyanosis, dyspnea, prostration, and death. The mice that survived were asymptomatic after 48 hr. The mice which died from an injection of tobacco infected with *A. tenuis* behaved differently according to the dose level. Those receiving high dosages died rapidly with the signs associated with nicotine. Those receiving lower dosage levels, near the LD_{50} value, died in about 24 to 48 hr and appeared to be depressed rather than stimulated. The main signs displayed by these mice, including survivors, were ataxia, lacrimation, and a mucoid defecation. The signs could intensify to the extent that the animals became prostrate, the eyes crusted over, and dehydration became so severe that Robichaud's sign appeared (4). The Robichaud test consists of picking up the loose skin of an animal's back and then suddenly releasing the skin fold. The skin of a normal animal will immediately readjust to the contours of the body. The test is considered positive if the perpendicular fold persists for 3 sec. Materials producing excessive diuresis, skeletal muscle relaxation, acidosis, or all three, produce this response. The delayed time of death and the physiological signs were duplicated by injecting the mice with homogenized pure cultures of *Alternaria* isolated from tobacco.

DISCUSSION

It should be emphasized that in the present tests toxicity was evaluated by ip injections. These rapid and inexpensive tests do not require elaborate equipment and are particularly suitable for preliminary studies that screen large numbers of fungi that may adversely affect the quality of tobacco. The data obtained with this test procedure show that a large proportion of the fungi isolated from tobacco before and after flue-curing are toxic when grown on baby cereal. High percentages of toxic isolates were found in the genera *Epicoccum*, *Alternaria*, *Chaetomium*, *Aspergillus*, and *Penicillium*. The apparent variations in toxicity among different isolates from the same genera or species may present difficulties in selecting the cultures and conditions to be used in further studies.

Samples of tobacco from three geographical locations infected naturally with *A. tenuis* appeared to produce physiological signs in mice which differed from those produced by comparable brown spot-free material. It will be recalled that Forgacs and Carll (2), Doupnik and Sobers (1), and Joffe (3) implicated members of the *Alternaria* in toxic conditions in a variety of animals including man. *Alternaria* then would seem to be a suitable candidate for more extensive studies to compare the effects of smoke or smoke condensates from moldy and nonmoldy tobacco on experimental animals. Such studies are in progress.

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