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Toxigenic Thermophilic and Thermotolerant Fungi

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Twenty-three isolates of fungi, representing 13 thermophilic and thermotolerant species, were bioassayed for toxigenicity to brine shrimp, chicken embryos, and rats. Thirteen isolates representing nine genera were highly toxic to at least two of the three systems. Seven additional isolates of five genera were slightly toxic.

Recent review articles attest to a rapidly growing literature in the field of mycotoxicology and to an increasing concern about the presence of toxigenic fungi in man's food supply (3, 7, 10-13). The importance of fungi as agents of decay in stored grains is well known (2). Stored foods, feeds, and grains that become moist and subsequently overheat may provide thermophilic fungi with a competitive growth advantage over other microorganisms. Despite the current interest in mycotoxicology, no toxic metabolites have been isolated from thermophilic fungi, even though *Thermomyces lanuginosus* has been implicated in bovine abortion and "Farmer's Lung" disease (8) and *Chaetomium thermophile* belongs in a genus that contains several mesophilic species that are toxigenic (11). We have found that at least one previously studied thermophile is potentially a toxin producer and that the group warrants investigation (unpublished data). Of the species used in this study, *Aspergillus fumigatus* is thermotolerant rather than thermophilic by most criteria and is a known toxin producer (4-6). This paper reports the results of an investigation to identify those thermophilic species that appear to produce mycotoxins.

MATERIALS AND METHODS

Organisms. Isolates of *A. fumigatus* (Auburn University Agricultural Experiment Station Collection [AUA] 772, 776), *Dactylomyces crustaceus* (AUA 774), *Mucor pusillus* (AUA 773), *Thermoascus aurantiacus* (AUA 770), and *Thermomyces lanuginosus* (AUA 771, 775) were obtained from corn samples, and *Talaromyces thermophilus* (AUA 1043) and *Thielavia albomyces* (AUA 1046) were obtained from cotton seed. *A. fumigatus* (AUA 805) and *T. thermophilus* (AUA 1044) were also isolated from hay and ground feed, respectively. The type isolate of *Acremonium alabamensis* from pine needles (AUA 758) and another from soil (AUA 759) were also investigated. Ten isolates of thermophilic fungi were obtained from the Quartermaster Culture Collection (QM), U.S. Army Natick Laboratories, through the courtesy of Emory G. Simmons. These included, in addition to some of the species listed above, *C. thermophile* var. *coprophile*, *Humicola grisea*, *Sporotrichum thermophile*, *Byssochlamys* sp., and *Torula thermophile*. Isolations were made by plating samples of natural substrates directly onto agar plates and incubating them at 50 C. Cultures were isolated and maintained on an agar medium containing 5% dextrose, 0.7% yeast extract (Difco), and 0.5% KH_2PO_4. Inocula were prepared from cultures of thermophilic fungi grown at 50 C by adding 20 ml of sterile water to test tube slants and making a suspension of spores and mycelium.

Culture of the organisms. Fungi were grown in 1-liter flasks on a medium composed of 50 g of shredded wheat and 100 ml of nutrient solution per liter flask. The nutrient solution consisted of 10% sucrose, 4% dextrose, 2% yeast extract (Difco), and 1% soytone. Flasks were stoppered with foam plugs and autoclaved for 15 min at 121 C. Flasks were autoclaved a second time 24 h later to ensure sterility. After inoculation, flasks were incubated 14 to 28 days at 48 ± 2 C at 98 to 100% relative humidity.

Preparation of extracts from moldy substrates. Approximately 100 ml of chloroform-ethanol solution (80:20) was added to each flask of moldy substrate, and the foam plug was replaced. Flasks were placed on a steam bath until solvent fumes were obvious and for several minutes thereafter to kill spores and mycelium as a safety precaution, since *A. fumigatus* in particular is a known human pathogen. Contents of each flask were transferred to a Waring blender jar (explosion proof), covered with 150 ml of additional solvent if necessary, blended for 1 min, and filtered through 33-cm rapid flow filter paper. The residue was reextracted in another 250-ml portion of solvent and filtered, and the two filtrates were combined. The solution was then filtered through no. 42 filter paper, and all residues were discarded. All filtrates thus prepared were concentrated to about 50 ml under an air stream at room temperature and divided into equal portions for the brine shrimp and the chicken embryo bioassays.

The extract for the brine shrimp bioassay was

completely dried under an air stream and taken up in 10 ml of 95% ethanol. The portion for the chicken embryo bioassay was transferred to a 50-ml flask containing 1.5 ml of peanut oil. The flask was plugged with cotton and heated on a boiling-water bath, permitting the chloroform-ethanol fumes to sterilize the flask and peanut oil. The flask was left on the water bath until most of the solvent evaporated, and the remaining solvent was then removed in a vacuum oven at 50 C for 24 h. Flask contents were maintained sterile by a cotton plug held in place with a small piece of cheesecloth and a rubber band. This extract suspended in the peanut oil carrier was used in the chicken embryo bioassay. Fungal extract-peanut oil suspension was similarly prepared with the contents of a second flask for the rat intraperitoneal bioassay.

Brine shrimp bioassay. The procedure used was essentially that described by Harwig and Scott (9), except that the duration of the bioassay was 4 h. Solutions contained a final concentration of approximately 4% ethanol in the fungal extract-seawater-brine shrimp mixture. This was prepared by adding 0.04 ml of extract in 95% ethanol to the artificial seawater-brine shrimp mixture in Pyrex 7220 chemical spotting plates to give a final volume of 1 ml. Controls consisted of the same mixture, with the extract being from uninoculated substrate. Plates were incubated at 27 to 29 C and 98 to 100% relative humidity.

Chicken embryo bioassay. Methods were those described by Verrett et al. (14) and by the Association of Official Analytical Chemists for aflatoxin bioassays (1). Locally obtained fertile chicken eggs were incubated 5 days until the blood vessels of the embryo were clearly visible on candling. Eggs were surface sterilized above the air sac with 5% iodine in 70% ethanol prior to puncture of the shell with a sterile 22-G needle before injection. Sterile disposable syringes were used to inject 0.1 ml of fungal extract-peanut oil suspension into each egg. For each extract, five eggs were inoculated in the sac and another five in the yolk. Eggs were incubated at 40 C and 84 to 86% relative humidity in a Leahy model 624 incubator. Eggs were turned three times daily, and data on embryo mortality were recorded after 4 days. Sac and yolk mortality data were combined.

Rat bioassay. Extracts were prepared as previously described. Charles Rivers (CD), 21-day-old weanling rats were given water but no food for the 24 h prior to treatment. Each rat was injected intraperitoneally with approximately 0.4 ml of fungal extract-peanut oil suspension. After injection rats were given food and water ad libidum. They were weighed before injection and again at the end of the 7-day experiment. Observations were made for several hours after injection, daily, and on autopsy after the 7th day. Weight gain or loss relative to the control group was calculated for each group of rats. Control rats were injected with the extract of uninoculated medium suspended in peanut oil.

RESULTS

Toxicity of thermophilic and thermotolerant fungi to brine shrimp, chicken embryos, and rats is shown in Table 1. Ten isolates representing eight genera were highly toxic to brine shrimp, causing 60 to 100% mortality in 4 h. Eighteen isolates (10 species) were highly toxic to chicken embryos, causing death of 50% or more of the embryos in 4 days. Nine isolates (six genera) were toxic to rats (intraperitoneally), as measured by a reduction in growth of 10% or more, in comparison to the controls for the 1-week period of the experiment.

C. thermophile var. *coprophile*, *Thermoascus aurantiacus*, and *Thermomyces lanuginosus* (two isolates) were consistently toxic to all three bioassay systems. Toxic to two of the three systems were: *Acremonium alabamensis*, *A. fumigatus* (two isolates), *D. crustaceus*, *M. pusillus*, *T. thermophilus*, *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, and *Torula thermophile*. *H. grisea* was toxic only to brine shrimp. Toxic only to chicken embryos were: *A. fumigatus*, *D. crustaceus* (two isolates), *Byssochlamys* sp., and *T. thermophilus*. One isolate of *T. thermophilus* was toxic only to rats, and one isolate each of *Acremonium alabamensis*, *S. thermophile*, and *Thielavia albomyces* was not toxic in any of the bioassays.

Various symptoms and disorders caused by the fungal extracts were noted during the rat intraperitoneal experiments. Extracts of *Thermomyces lanuginosus* (isolates 757 and 775) appeared to cause a slight ataxia. Extracts of *Bussochlamys* sp. and *C. thermophile* var. *coprophile* seemed to cause reduced fecal output. Isolates of several fungi (757, 770, 771, 772, 773, 805, 1043, 1044, and 775) appeared to cause a slight granulomatous condition in the livers and kidneys in comparison to control rats. Two of five rats in one experiment died in 3 days from intraperitoneal injections with extracts of *Thermomyces lanuginosus* isolate 775. One of five rats died in 1 day when injected with *Thermomyces lanuginosus* isolate 771.

DISCUSSION

No toxigenicity studies have been reported for thermophilic fungi. Of 23 fungus isolates in this investigation, *A. fumigatus* is probably thermotolerant rather than thermophilic. This fungus is known to be toxigenic and provided a basis for comparison in addition to untreated controls. With the exception of *S. thermophile*, *Thielavia albomyces*, and one isolate of *Acremonium alabamensis*, all of the fungi tested had some isolates that must be considered potential mycotoxin producers. The genus *Chaetomium* has at least three nonthermophilic species that form toxic metabolites (11), in addition to *C. thermophile* var. *coprophile* which was found to be toxigenic in this study. The possibility that

TABLE 1. *Toxicity of extracts from isolates of 13 thermophilic and thermotolerant fungi to brine shrimp, chicken embryos, and rats*

Culture no.		Fungus	Brine shrimp mortality[a]	Chicken embryo deaths/ total eggs	Rats, % wt loss[b]
AUA collection	QM collection				
None		Uninoculated checks	0	1/20	0
758		*Acremonium alabamensis*	0	9/20	6
759		*A. alabamensis*	1	12/20	45
772		*Aspergillus fumigatus*	0	11/20	10
776		*A. fumigatus*	2	12/20	4
805		*A. fumigatus*	1	14/20	4
751	9381	*Chaetomium thermophile* var. *coprophile*	2	7/10	17
760	6798	*D. crustaceus*	0	10/20	0
763	6878	*D. crustaceus*	1	10/20	3
774		*D. crustaceus*	2	15/20	2
753	228	*H. grisea*	2	7/20	(+6)[c]
773		*M. pusillus*	2	13/20	0
755	9382	*S. thermophile*	0	2/10	0
750	438	*Byssochlamys* sp.	0	10/20	(+3)[c]
754	1851	*Talaromyces thermophilus*	0	7/10	2
1043		*T. thermophilus*	0	0/10	30
1044		*T. thermophilus*	0	5/10	37
756	9383	*Thermoascus aurantiacus*	0	17/20	10
770		*T. aurantiacus*	2	11/20	20
1046		*Thielavia albomyces*	0	2/10	(+7)[c]
757	225	*Thermomyces lanuginosus*	2	12/20	24
771		*T. lanuginosus*	2	12/20	45[d]
775		*T. lanuginosus*	2	13/20	0[d]
752	1192	*Torula thermophile*	2	5/10	3

[a] 2, 60 to 100% mortality of shrimp; 1, 20 to 59% mortality; and 0, 1 to 19% mortality (approximately 50 brine shrimp/ml).

[b] Average for five rats per treatment.

[c] Weight gain relative to uninoculated checks.

[d] Rat mortality; two died in 3 days from culture 775 and one died in 1 day from culture 771.

thermophilic fungi are mycotoxin producers is of significance to human and animal health. Standard methods of mycofloral analysis will not reveal their presence and their metabolites are generally unknown. Thus, the presence of thermophilic fungi in grain, food, and feed may present a hitherto unrecognized hazard to man. Certainly the possibility warrants further research.

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