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Separation of Mycotoxin-Containing Sources in Grain Dust and Determination of Their Mycotoxin Potential

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Two distinct reservoirs of mycotoxins exist in fungal-infected cereal grains—the fungal spores and the spore-free mycelium-substrate matrix. Many fungal spores are of respirable size and the mycelium–substrate matrix can be pulverized to form particles of respirable size during routine handling of grain. In order to determine the contribution of each source to the level of mycotoxin contamination of dust, we developed techniques to harvest and separate mycelium–substrate matrices from spores of fungi. Conventional quantitative chromatographic analyses of separated materials indicated that aflatoxin from *Aspergillus parasiticus*, norsolorinic acid from a mutant of *A. parasiticus*, and secalonic acid D from *Penicillium oxalicum* were concentrated in the mycelium–substrate matrices and not in the spores. In contrast, spores of *Aspergillus niger* and *Aspergillus fumigatus* contained significant concentrations of aurasperone C and fumigaclavine C, respectively; only negligible amounts of the toxins were detected in the mycelium–substrate matrices of these two fungi.

Introduction

Cereal grains may become contaminated with fungi and products of their secondary metabolism either in the field or during storage. Fungal mycelia infect grain by infiltrating the substrate with a mass of hyphae at the same time secreting hydrolytic enzymes that partially degrade the substrate. The resultant "koji" is a heterogeneous, sometimes crumbly mass of mold and substrate (*1*). This mycelium–substrate (MS) matrix can be pulverized to form particles of respirable size by the friction caused in the transfer and handling of dry grain. In a report of aflatoxin concentration in the friable material broken from cottonseed during dehulling, spores were observed on the surface of the meats just under the hulls (*2*). Microscopic examination of a single peanut provided evidence of fungal mycelia throughout the substrate with spores easily visible in the air-space near the center (*3*). The presence of many scattered spores of respirable size (< 10 μm) on the outer surface of grains indicates that in nature spores exist separate from the MS matrix. Both spores and dust pose a health hazard to handlers of grain throughout harvesting, shipping and storage.

Products of secondary fungal metabolism, often toxic and termed mycotoxins, can be located within the hyphae, spores (conidia), or excreted into the substrate (grain). Very little evidence exists to indicate the amount of mycotoxins in spores as opposed to the amount exuded into the substrate or in hyphae imbedded in the substrate. Wicklow and Shotwell (*4*) demonstrated aflatoxin B_1 at concentrations up to 97,000 and 135,000 ppb in spores of *A. flavus* and *A. parasiticus* respectively. Previous reports indicate that spores of *A. parasiticus* and *A. flavus* contained aflatoxins, but toxins were not quantitated (*5,6*). Aflatoxins have also been reported in the mycelia of both *A. parasiticus* and *A. flavus* (*5*). Although mycotoxin contents of spores from other fungi have not been reported, there are reports of mycotoxins in the entire mycelium, including the spores or the substrate, or both. The substrate of *Penicillium oxalicum* contained negligible amounts of secalonic acid (SAD), while large amounts were detected in the mycelium (*7*). Neurotoxic naphtho-γ-pyrones have been reported in cultures of *Aspergillus niger* (*8*), but spores were included with the rest of the mycelium when the cultures were examined for mycotoxins.

The contribution of either spores or the mycelium–substrate (MS) matrix to mycotoxin contamination is governed by the distribution of toxin between spores and mycelium. In the present report a technique was developed to effectively separate fungal spores from their MS matrices in order to facilitate mycotoxin quantitation in the separated parts. Cultures of four fungi identified as contaminants of grain (*9*) and a colored mutant were examined for the distribution of mycotoxins.

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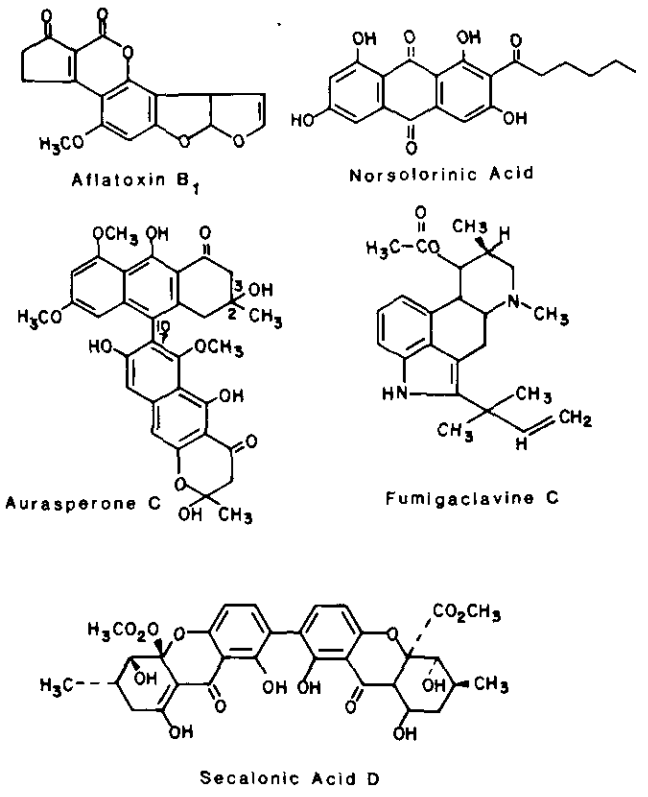
STRUCTURES OF MYCOTOXINS



Materials and Methods

Preparation and Separation of Fungal Components

Fungi were inoculated onto 100 g of autoclaved rice, adjusted to 42% moisture, and incubated for 7 days at 25°C. Crystallizing dishes sealed with aluminum foil and a foam rubber plug were used as culture dishes to facilitate sample removal. Five strains were used as separate cultures: *Aspergillus parasiticus* (SRRC-2004); a mutant of *Aspergillus parasiticus*, NOR-1 (SRRC-162); *Aspergillus niger* (SRRC 2005); *Aspergillus fumigatus* (SRRC-2006); and *Penicillium oxalicum* (SRRC-2007). Three dishes of rice were inoculated for each fungus. Foil covers were replaced with cheesecloth prior to oven-drying of cultures at 50°C for 3 days. A moisture determination was made on the dried material. Duplicate 10-g aliquots of dried cultured rice were removed from each dish for mycotoxin quantitation on each of three fractions to be further separated into spores, MS matrix, and MS matrix plus spores (unseparated).

Spores. Two 10-g aliquots of each rice culture were placed separately into a beaker enclosed in a plastic bag. As the culture was agitated, air, laden with spores was drawn out of the bag and filtered through a membrane filter (0.45 μm pore size) by vacuum. Some spores remained with the MS matrix; no attempt was made to harvest all the spores. A small portion of the collected material (< 1 mg) was removed from the filters, weighed, suspended in a Tergitol solution, and the number of spores determined with a hemacytometer chamber. Fields were searched for absence of hyphal fragments. Spores trapped on the filter were weighed, transferred to a glass-stoppered bottle containing 0.45 mm glass beads and water, and disrupted in a Braun homogenizer. The aqueous extract was partitioned twice in a separatory funnel with methylene chloride. The methylene chloride extract was filtered through sodium sulfate, the sulfate was washed with additional methylene chloride, and the resulting extract evaporated under reduced pressure. Each extract was transferred to a glass vial fitted with a Teflon-lined cap, dried under a stream of nitrogen, and reserved for quantitation by thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC).

MS Matrix. Duplicate 10-g aliquots from each rice culture were placed in a beaker covered with cheesecloth secured with a rubber band. The culture was subjected to a severe blowing technique in which the MS matrix and spores were blown about in the beaker by air at 125 mph from a modified commercial leaf blower. Blowing was continued until rice was visibly free of spores. The MS matrix was weighed and extracted with methylene chloride by soaking overnight. Extracts were handled in the same manner as those made from spore preparations.

MS Matrix and Spores. Duplicate 10-g aliquots from each culture were extracted with methylene chloride in the same manner as that used for the MS matrices above.

Mycotoxin Analyses

Extracts were made to a known volume in benzene:acetonitrile (98:2) and chromatographed according to standard procedures for each toxin. Aflatoxin B_1 from the *A. parasiticus* culture, norsolorinic acid from the mutant of *A. parasiticus* culture, and fumigaclavine C from the *A. fumigatus* culture were quantitated by TLC according to standard procedures (*11,12,13*). Since quantitative standards were not available for *A. niger* metabolites, two additional 100-g portions of rice were inoculated with *A. niger* and extracted with methylene chloride. The extract was dried with sodium sulfate and the extract subjected to preparative TLC in solvent systems used by Ehrlich et al. in their separation of naphtho-γ-pyrones from *A. niger* cultures (*14*). The major yellow-pigmented band contained a material that had chromatographic properties identical to those of aurasperone C (*14*) when compared to a sample of mixed aurasperones supplied by these authors. The material from this band was further purified by preparative TLC and was used as a standard in quantitating aurasperone C in the *A. niger* cultures. Secalonic acid from *P. oxalicum* cultures was quantitated by HPLC (*15*).

Results and Discussion

Mycotoxins structures are shown in the figure. Mycotoxin distribution in the three fractions: spores, MS

Table 1. Weights of fungal matrices and spores and mycotoxin content of separated fractions.

Fungus	Toxin	Matrix and spores		Matrix		Spores	
		Sample wt, g	Toxin in sample, μg	Sample wt, g	Toxin in sample, μg	Sample wt, g	Toxin in sample, μg
A. parasiticus	Aflatoxin B_1	10	2420	9.2	2250	0.9	145
A. parasiticus mutant, Nor-1	Norsolorinic Acid	10	7350	9.2	7000	0.6	trace
A. niger	Aurasperone C	10	1100	9.8	16	0.1	937
A. fumigatus	Fumigaclavine C	10	390	9.0	12	0.7	450
P. oxalicum	Secalonic Acid D	10	4880	8.7	4140	0.3	2

matrix, and spores plus matrix are reported in Table 1. Values are based on 10 g sample with no correction for moisture. Moisture contents of the dried cultures were all between 4 and 6%. No attempt was made to distinguish between the location of the toxin either in hyphae at the surface or hyphae deep within the substrate. Since friction or abrasion produces particles of respirable size from either portion, it was not necessary to determine the exact location of the toxin within the MS-matrix. Our purpose was to separate spores from the MS matrices and determine the mycotoxin concentration in each part. Distribution of toxin in spores and the MS matrices varied with the fungus. Major concentrations of the mycotoxin secalonic acid D occurred in the MS matrix of the *P. oxalicum* culture with only trace amounts detected in the spores. Similarly, concentrations of the red-pigmented norsolorinic acid were much greater in the MS-matrix plus spores and the MS-matrix than in the spores alone. Only trace amounts were detected in spores. It is of interest that in the parent strain, *Aspergillus parasiticus*, from which the mutant producing norsolorinic acid was derived (*12*), a substantial amount of aflatoxin B_1 was detected in the spores, although the amount was much less than that in the MS matrix. The mutation not only changed the mycotoxin produced but its location in the spore-hyphal association. In contrast, spores of *A. niger* and *A. fumigatis* contained nearly all of the toxins; 937 μg of aurasperone C was detected in spores harvested from 10 g of *A. niger* culture, and 450 μg of fumigaclavine C was detected from spores of *A. fumigatus*. It was difficult to remove all spores from the rice substrates even by the severe blowing technique. Older conidia were detached from spore heads much more readily than were immature conidia. Because of this strong attachment the blowing technique removed more spores from the MS matrix than did the vacuum technique. Mycotoxin concentrations reported for spores may be slightly affected by toxins in contaminating mycelial fragments; conversely, values for the MS matrix may be slightly affected by contaminating spores. However, no mycelial fragments were observed attached to the spores removed for the spore count. Attachment of mycelium to substrate was strong. The severe wind-blowing technique that was necessary to separate spores from their mycelium did not remove the mycelium from the substrate. Removal of the spores in most cases was fairly clean. However, a few spores were observed deep between grains of rice even after the 125 mph wind treatment. Hyphae from the *A. niger* culture still strongly attached to the substrate were translucent white when the intensely black spores were blown off, a visual confirmation of mycotoxin location. Cursory observation of the highly red-pigmented mutant, NOR-1, confirmed chemical analyses indicating that this metabolite was mycelium oriented. The MS matrix was brick-red, while the spores were brownish. Neither the distribution of aflatoxin B_1, fumigaclavine C, nor secalonic acid D could be predicted by color of the spores or substrate.

Previous reports of toxin concentrations in spores did not relate the number of spores per gram to the toxin levels for a given number of spores. Our results provide information on the number of spores per gram and toxin levels for a given number of spores (Table 2). Since spore counts are a routine part of dust analyses, data such as ours are necessary to determine the magnitude of toxin contamination as related to spore counts. Since aflatoxins are produced by *A. parasiticus* and are among the most potent carcinogens, both the distribution of aflatoxin B_1 between spores and the MS matrix and the content of this toxin in spores is important. While 10^6 spores contain just 1 ng of aflatoxin B_1, the *A. parasiticus* MS matrix contains 16 times that amount (Table 1).

Clearly, when mycotoxins are contained within spores, a much greater probability of exposure for workers handling moldy grain would be expected because the spores are more easily suspended in the air and

Table 2. Spore counts and toxin concentrations.

Fungus	Spores/g	Toxin	Toxin concentration	
			ng/g of spores	ng/10^6 spores
A. parasiticus	2.2×10^{10}	Aflatoxin B_1	16,600	0.976
A. parasiticus Mutant Nor-1	1.22×10^{10}	Norsolorinic acid	280	0.023
A. niger	4.0×10^{10}	Aurasperone C	460,000	0.114
A. fumigatus	9.4×10^{10}	Fumigaclavine C	930,000	9.89
P. oxalicum	7.5×10^{10}	Secalonic acid D	1,890	0.025

remain suspended for long periods of time. The mycotoxins retained in the MS matrix would pose a problem when the MS matrix becomes pulverized. For toxins present in the MS matrix, secalonic acid D and aflatoxin, the larger quantity of toxin was in the MS matrix and could pose a considerable hazard if dust were produced from heavily contaminated grains.

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