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Comparative study of *Aspergillus mycotoxin* production on enriched media and construction material

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Isolates of *Aspergillus flavus* and *Aspergillus fumigatus* from indoor air were compared with a known mycotoxin producer for their capacity to produce mycotoxins on a variety of enrichment media and with growth on indoor substrates such as ceiling tile and wall board. In enrichment media, four of seven isolates of *A. flavus* produced at least one aflatoxin and both isolates of *A. fumigatus* produced mycotoxins. The spectrum of mycotoxins and their concentrations varied with the strain and medium. When the mycotoxin-positive strains were grown to a dense concentration on indoor construction and finishing materials such as ceiling tile and wall boards, mycotoxins were not detected in extracts of the materials. Colonization of indoor surfaces by mycotoxin-producing strains of *A. flavus* and *A. fumigatus* may not necessarily expose inhabitants to mycotoxins or result in production of mycotoxins.

Keywords: *Aspergillus*; mycotoxin; ceiling tiles

Introduction

Mycotoxins are secondary metabolites produced by fungi that pose a hazard to the health of humans and animals. These compounds are noted for their toxicity and extreme carcinogenicity in test animals. Recent reports suggest that mycotoxins may play a role in the symptoms experienced by those occupants of buildings that are heavily contaminated by certain fungal species [10,18,33].

Indoor air quality is a matter of growing concern in environmental and occupational health [27]. Allergic and hypersensitivity reactions due to microbial allergens are thought to contribute to the adverse health effects called sick building syndrome (SBS) [4].

The tremorgenic mycotoxins—fumitremorgen and verruculogen, as well as helvolic acid, fumagillin, and gliotoxin are commonly isolated from *Aspergillus fumigatus*. Mycotoxins are usually ingested with tainted foods but may be absorbed through the mucus membranes of the respiratory tract as well [32,34,37]. Several studies have demonstrated that airborne particulates such as dust, mycelial fragments and conidia contain mycotoxins. Concentrations of aflatoxin B_1 up to 612 ppb have been reported in airborne dusts arising from handling or processing of contaminated corn and peanuts [6,31,35]. Once absorbed, the systemic effects may be similar to, or even greater than, those of mycotoxins absorbed through digestion or other routes [36].

The aspergilli are metabolically diverse and are frequently isolated from the indoor environment. *Aspergillus flavus* is found on a variety of substrates including foods, wood and wood pulp, birds' nests, leather, cotton and building materials [3]. *A. fumigatus*, a thermotolerant species which grows well at 45°C, is a successful saprophyte that thrives in self-heating compost piles [26]. It has been reported from air, furniture, dust, cellars, soil of potted plants, condensation from refrigeration appliances, and foodstuffs such as ground coffee, powdered milks, and spices [25]. The number of *A. fumigatus* conidia present in air usually ranges from 0.1 to 10 conidia m^{-3}, but may reach 10^6 conidia m^{-3} under certain environmental conditions [5,15,23]. Due to their small size (2–3 μm) and presence in the air, *A. fumigatus* conidia are inhaled by all individuals and travel through the upper respiratory tract, bronchia and bronchioles to end in the alveoli [7].

The objective of this study was to evaluate the ability of several isolates of *A. flavus* and *A. fumigatus* from indoor air or substrates to produce mycotoxins on building and finishing materials such as ceiling tiles, wallpaper, and air-conditioning filters.

Materials and methods

An aflatoxin-positive strain of *Aspergillus flavus* NRRL-3251 was obtained from the National Center for Agricultural Utilization Research (Peoria, II, USA). Isolates of *A. flavus* 920617, 930206, 930601, 930902, and 931014 and two isolates of *A. fumigatus* 930225 and 930421 were obtained from indoor air samples collected with a single-stage Andersen air sampler (1 min at a flow of 1 cfm). Various urban residential and commercial sites were sampled. Fungi developing on the enumeration media were identified by classical morphological features [8]. *A. flavus* F1 was isolated from the acrylic latex facing of fiberglass insulation material from a heating, ventilation and air-conditioning (HVAC) system and *A. flavus* CT was obtained from a cellulosic air filter frame from a commercial building in metropolitan Atlanta, GA. Stock cultures were maintained on corn meal agar (CMA, Difco, Detroit, MI, USA) slants at 4°C and were transferred bimonthly to fresh medium.

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Fungi were grown on potato dextrose agar (PDA, Difco) in petri plates (9 cm) for approximately 5 days at 25°C. The plates were then flooded with sterile 0.9% saline containing 0.5% Tween 80 (10 ml per plate). The conidia were gently dislodged with a sterile pipette and the resulting suspensions were filtered through sterile glass wool to remove fragments of mycelia. The suspension of conidia was adjusted to give a density of approximately 10^6 conidia ml^{-1}. Broth cultures (100 ml in a 250-ml Erlenmeyer flask) were inoculated with 1 ml of the conidial suspension and solid media with 100 μl per plate.

Fungal isolates were screened for mycotoxin production in solid and liquid media [13]. The following media were used to screen for aflatoxin production in *A. flavus* isolates: Czapek yeast autolysate agar (CYA) [28], glucose yeast extract agar (GYA) [9], yeast extract sucrose agar (YES) [12], malt extract agar: (MEA, Difco) [28], basal medium agar (BMA) [17], cellulose agar (yeast extract, 0.5 g; peptone, 0.5 g; cellulose, 10 g; agar, 15 g; H_2O, 1000 ml), and simplified nutritional agar (SNA) [24]. *A. fumigatus* isolates were screened on CYA. Liquid media with similar compositions were used to compare the production of toxins to fungal dry weight. All culture systems were extracted after 14 days incubation in the dark at 28°C.

The fungal cultures grown on solid media (eight Petri dishes) were extracted with 150 ml of chloroform-methanol (2:1, v/v) in a homogenizer at high speed for 30 s and the slurry was filtered through a Whatman No. 1 filter paper. After addition of 1 ml of 85% phosphoric acid, the culture suspension was extracted again with acetone-ethyl acetate (1:1, v/v) in a blender for 15 s and the suspension was drawn through a Whatman No. 1 filter paper. Both fractions from the first and second extractions were refiltered through a Whatman 1PS phase separator filter paper, and the organic phase was evaporated to dryness in a Büchi rotary evaporator under reduced pressure at 55°C. After the residue had cooled it was redissolved in 3 ml of methanol. The methanol solution was filtered through a disposable 0.45-μm filter prior to analysis by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) [14]. Following filtration to separate the mycelial mass, mycotoxins were extracted from the broth cultures and mycelia with methanol and chloroform as described [30]. The weight of dried mycelium was determined gravimetrically after treatment at 80°C for 24 h.

Suspensions of conidia (10^6 conidia ml^{-1} in 200 ml of saline Tween) from PDA were extracted three times with 100 ml of chloroform. The extract was filtered through Whatman No. 3 filter paper and evaporated to dryness under reduced pressure at 55°C on a rotary evaporator. The residue was dissolved in 3 ml of methanol for chromatographic analysis. All experiments were performed in triplicate.

Building materials were inoculated with conidia to study the production of mycotoxins under simulated environmental conditions. Ceiling tiles, wallpaper without a plastic cover and air filters used in HVAC systems were purchased retail. Ceiling tiles were cut into sections about 7×10 cm^2 and other materials about 5×7 cm^2 sections. Some sections of ceiling tile were autoclaved at 120°C for 1 h and dried. Before inoculation, the ceiling tiles were submerged in sterile water for 1–5 min. Sections of building materials were inoculated with conidial suspensions (10^6 conidia ml^{-1}) and positioned within sealed vessels with concentrated salt solutions designed to hold relative humidities at 78–81%, 84–89%, and 97–100% [11]. The vessels were held at 28–30°C for at least 4 weeks in the dark. All inoculated materials were examined microscopically for growth following incubation. The densely colonized materials were transferred to sterile glass beakers containing 200 ml of chloroform-methanol (2:1, v/v). The beakers were sealed and agitated for 24 h. These crude organic extracts were filtered through a disposable 0.45-μm pore size filter and stored at 4°C for TLC and HPLC analysis.

Supplemental carbon or nitrogen sources including CY broth, 3% sucrose, 2% glucose, 0.5% yeast extract or 0.3% $NaNO_3$ solutions were added separately to the building substrates. These pretreated materials were flooded with the 1.0–2.0 ml of conidia suspension (10^6 conidia ml^{-1}) and incubated in environmental chambers under the conditions described above.

Qualitative analysis by thin-layer chromatography (TLC)

Extracts from cultures were screened by TLC on Merck precoated silica gel G, art. 5721 plates with chloroform-acetone (9:1, v/v) as the developing solvent. Ten microliters of each sample were loaded on the gel and the eluent was allowed to migrate at least 15 cm from the application line. The air-dried plates were examined under long wavelength UV light for aflatoxins. Toluene-ethyl acetate-90% formic acid (5:4:1) was used as developing solvent with extracts of *A. fumigatus*. The toxins were visualized with 50% sulphuric acid, followed by 5–10 min at 110°C, and visualization with short wavelength UV. Methanol solutions of standard mycotoxins (0.2 mg ml^{-1}) aflatoxin B_1, B_2, G_1, G_2; verruculogen, helvolic acid, and fumagillin (Sigma, St Louis, MO, USA) were used as standards for comparison of fluorescence and mobility.

High-performance liquid chromatography (HPLC)

Fifty microliters of extract were injected into a Perkin-Elmer ISS-200 Advanced LC sample processor (Perkin-Elmer Corp, Norwalk, CT, USA). The samples from *A. flavus* cultures were pumped with a binary LC pump (Model 250) through a Prodigy™ 150×4.6 mm I.D. reversed-phase 5-μm C8 Column (Phenomenex, Torrance, CA, USA). Methanol:water (45:55, v/v), degassed with helium, was used as mobile phase at a flow rate of 0.8 ml min^{-1}. Detection was accomplished with a Perkin-Elmer LC-135C/LC-235C diode array detector (DAD) at 220 nm. Verruculogen was analyzed with a Prodigy™ 150×4.6 mm I.D. reversed-phase 5-μm ODS(2) analytical column (Phenomenex). The mobile phase was methanol:water (72:28, v/v) at a flow rate of 2.0 ml min^{-1}. DAD was at 210 nm. Helvolic acid and fumagillin were assayed on a Phenosphere™ 150×4.6 mm I.D. reversed-phase 5-μm ODS(1) column (Phenomenex). Methanol:water (95:5, v/v) at a flow rate of 1.0 ml min^{-1} was used as mobile phase for helvolic acid detection and methanol:water (85:15, v/v) at 2.0 ml min^{-1} for fumagillin detection. Helvolic acid was detected at 235 nm and fumagillin was detected at 330 nm.

Table 1 Amount of aflatoxins (B_1 and B_2) produced in broth cultures and present in conidia

Strain/isolate *A. flavus*	GY		CY		YES		ME		BM	
	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
NRRL-3251	868.7 ± 17.5[a]	642 ± 16	1028 ± 23	627 ± 22	178 ± 8	134 ± 20	1018 ± 12	1183 ± 41	911 ± 21	575 ± 34
920617	836.3 ± 32.8	765 ± 17	1147 ± 39	573 ± 6	71 ± 8	73 ± 4	823 ± 29	658 ± 16	738 ± 44	686 ± 31
930601	649.3 ± 15.9	468 ± 11	821 ± 42	321 ± 36	136 ± 28	69 ± 6	726 ± 39	+/–	715 ± 18	+/–
930902	700.5 ± 6.7	512 ± 27	762 ± 20	273 ± 24	146 ± 28	66 ± 6	798 ± 29	+/–	699 ± 31	+/–
931014	734.1 ± 31.6	267 ± 11	914 ± 23	423 ± 24	147 ± 9	–	866 ± 24	–	841 ± 24	–
CT	525 ± 12.5	247 ± 11	430 ± 21	250 ± 28	110 ± 22	–	618 ± 18	–	426 ± 12	–
930206	–[b]	–	–	–	–	–	–	–	–	–
FI	–	–	–	–	–	–	–	–	–	–
A. flavus conidia										
NRRL-3251	48 ± 3.6[c]	3.6 ± 1.4	18.2 ± 5.6	1.03 ± 0.2	0.15 ± 0.08	–	0.29 ± 0.1	–	24 ± 1.9	1.50 ± 0.3
931014	0.130 ± .025	–	3.3 ± 0.6	–	0.04 ± 0.02	–	0.03 ± 0.02	–	0.09 ± 0.03	–

[a] $\mu g\ g^{-1}$ of dry mycelium, $n = 3$.
[b] No aflatoxin detected.
[c] $\mu g\ 10^{-8}$ conidia, $n = 3$.

Toxins were characterized and quantified from comparisons of retention indexes and on-line UV spectra obtained from standard solutions.

Results

Five isolates of *A. flavus* produced aflatoxin B_1 on all media and aflatoxin B_2 only in Gy and CY broth cultures (Table 1). The concentrations varied with the broth composition. Only one isolate, strain 920617, produced both aflatoxins in all enrichment broths. The same media prepared with agar gave similar results with B_2 being found at a slightly lower frequency (data not presented). Two isolates, 930206 and F1, produced no aflatoxins under any culture condition tested. Conidia of two representative strains were shown to contain mainly aflatoxin B_1 with aflatoxin B_2 absent or in insignificant concentrations. Growth on cellulose and simplified nutritional agar did not result in aflatoxin production.

No mycotoxins were found in extracts of densely colonized ceiling tiles, wall board, wallpaper and air filters. These negative results were obtained even with enhanced growth when the indoor construction and finishing materials were supplemented with carbon and nitrogen.

Both isolates of *A. fumigatus* produced verruculogen, helvolic acid and fumagillin when grown in CY broth (Table 2). The three mycotoxins were detected in the conidia of isolates 930225 but only verruculogen and helvolic acid were detected in conidia of isolate 930421. As with *A. flavus*, mycotoxins were not detected in extracts of fungal growth on any of the indoor materials.

Discussion

Aflatoxin production by different isolates of *A. flavus* varied with growth conditions. Aflatoxin B_1 was produced on most of the media, whereas production of aflatoxin B_2 was more restricted. Although *A. flavus* grew best in YES medium, aflatoxin production was greater in other media which contained higher carbohydrate (ie sucrose or glucose) concentrations. However, concentrations (over 15%) of glucose were not necessary for high (above 500 μg toxin g^{-1} of dry mycelium) production of aflatoxin. Similar findings have been reported previously [1,2].

Zinc has been reported to be an important trace metal for production of aflatoxin. Mateles and Adye [21] found that a minimum of 0.4 ppm (0.4 mg L^{-1}) of zinc in the medium was required for aflatoxin production. Gupta *et al* [16] stated that addition of 10 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ to the medium induced the highest aflatoxin production. The CY medium used in this study which contained 10 mg L^{-1} $ZnSO_4 \cdot 7H_2O$ was the best medium for producing aflatoxins.

Factors controlling *in situ* production of aflatoxins and other mycotoxins are poorly understood. Although *A. flavus* and *A. fumigatus* usually grew well on cellulosic gypsum board and ceiling tiles at higher RH levels (97–100%), no significant levels of aflatoxin, verruculogen, helvolic acid or fumagillin were detected. Furthermore, mycotoxin production was greater with chemically defined carbohydrate-rich media, but mycotoxins were not detected when additional carbon or nitrogen sources were added to building and finishing materials. The possibility that toxins were present and bound by components in the substrates hindering their detection cannot be ruled out. We have demonstrated toxin production on building and finishing materials by isolates of *Alternaria alternata* [29]. Sorenson *et al* [35] demonstrated the presence of mycotoxins in environmental samples from a residence extensively contaminated with *Stachybotyrs chartarum*.

Strains of *A. fumigatus* are capable of producing a large number of potent mycotoxins including verruculogen, helvolic acid and fumagillin. The presence of toxins in conidia of other species of *Aspergillus* has been reported. Land *et al* [20] reported that the tremorgens fumitremorgen and verruculogen in conidia of *A. fumigatus* ranged from 6–80 μg 10^{-9} conidia. In the present study, the total mycotoxin concentration in conidia of both strains of *A. fumigatus* fell within this range. This fungus produces large amounts of small and easily dispersed conidia which may lead to exposure to tremorgenic mycotoxins by inhalation. Mycotoxins absorbed through the mucus membranes of the respiratory tract may play a significant role in aflatoxicoses [36]. Since aflatoxins can penetrate the skin [19], dermal uptake of mycotoxins is also a possibility. These toxins are potentially part of mold dust in the environment and may present a significant health risk to humans.

While conidia growing on chemically defined media have significant concentrations of aflatoxin, our data do not indicate that building and finishing materials support such production. However, conidia may germinate and grow in tissue potentially resulting in secondary mycotoxicoses. In a patient diagnosed with aspergillosis, Mori *et al* [22] found aflatoxin produced in lung tissue colonized by an isolate of *A. flavus*.

Table 2 Concentration of *Aspergillus* toxins produced by *A. fumigatus* in CY broth

	Isolates	Verruculogen	Helvolic acid	Fumagillin
Broth	930225	290 ± 10[a]	228 ± 11	203 ± 11
	930421	270 ± 11	226 ± 7	349 ± 17
Conidia	930225	1.01 ± 0.07[b]	0.756 ± 0.11	0.7 ± 0.1
	930421	0.57 ± 0.1	0.44 ± 0.10	–[c]

[a] μg g^{-1} of dry mycelium, $n = 3$.
[b] μg 10^{-8} conidia, $n = 3$.
[c] Not detected.

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