

## A STUDY OF AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* GROWING ON WALLBOARD

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### ABSTRACT

The potential for exposure to mycotoxins in indoor environments is of increasing concern. In order to evaluate the potential for mycotoxin production by toxigenic fungi growing on water-damaged building materials, two aflatoxin producing strains of *Aspergillus flavus* (American Type Culture Collection 16875 and 15547) were inoculated onto culture media, plain wallboard, and vinyl wallpapered wallboard (cellulose-based and wheat-based wallpaper paste) and incubated at high relative humidity and room temperature for up to 16 weeks. Each sample was extracted with 60% methanol and aflatoxins in the crude extract were collected by immunoaffinity chromatography and quantified by fluorometry. Analysis by high performance liquid chromatography was performed for confirmation. Varying degrees of fungal growth were evident on all tested substrate types. Up to 4800 ppb of aflatoxin was detected when strain ATCC 16875 was grown on potato dextrose agar. However, when inoculation was standardized to minimize initial aflatoxin concentration in the inoculum, aflatoxin production was not detected on any wallboard sample under any of the incubation conditions provided. The presence of a toxigenic fungal strain on an indoor substrate does not necessarily indicate that the fungus is producing mycotoxins and our data provide evidence that wet wallboard is unlikely to provide appropriate conditions for aflatoxin production.

### INTRODUCTION

Mycotoxins are secondary fungal metabolites with well-known health effects including carcinogenesis, immunosuppression, and cytotoxicity (Corrier, 1991; Hayes et al., 1984; Hendry and Cole, 1993; Jakab et al., 1994; Sorenson et al., 1986; Tobin et al., 1987). The growth in indoor environments of fungi that are known toxin-producers is of increasing concern. Mycotoxins have been found in spores, fungal hyphae and substrate materials, all of which could be released into the air when toxigenic fungi colonize and sporulate on indoor building materials (Burg et al., 1981; Croft et al., 1986; Flannigan, 1987; Hendry and Cole, 1993; Jarvis, 1990; Morey, 1993; Palmgren and Lee, 1986; Pasanen et al., 1993; Sorenson et al., 1987; Sorenson et al., 1981; Wicklow et al., 1982). This raises the possibility of potentially hazardous exposures to inhabitants of contaminated spaces.

Historically, mycotoxin research has focused on the health effects resulting from ingestion exposure and intense inhalation or dermal exposures in agricultural and industrial settings (Dvorackova, 1976 and 1986). However, the potent effects demonstrated in laboratory animals and conclusions derived from anecdotal indoor environment case studies have caused concern regarding mycotoxin exposure in normally clean indoor environments. There is experimental evidence of fungal growth and toxin production on building materials (Nikulin et al., 1994). However, a definitive causal relationship between indoor exposures to toxigenic fungi and

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### KEY WORDS

aflatoxin, wallboard, *Aspergillus flavus*, mold, fungi

adverse health effects has not been established. Some of the health effects that have been attributed to acute mycotoxin inhalation exposure include symptoms associated with indoor air quality complaints, such as headaches, malaise, and cough (Emmanuel, 1975). Data on the effects of chronic low level inhalation exposure, which is what one would expect in most indoor environments, are limited.

Aflatoxins are a group of mycotoxins produced by some species of *Aspergillus flavus* and *Aspergillus parasiticus*. The carcinogenic effects and acute toxicity of aflatoxin have been demonstrated by human cell culture experiments and industrial epidemiological and anecdotal studies (Coulombe et al., 1991; Dvorackova, 1976; Hayes et al., 1984; Olsen et al., 1984; Wilson et al., 1990). Many papers examining the potential risks associated with the presence of mycotoxins in indoor environments discuss *Aspergillus flavus* and the potent health effects from inhalational exposure to aflatoxins (Flannigan et al., 1991; Hendry and Cole, 1993; Miller, 1992; Smith et al., 1992; Tobin et al., 1987). Epidemiological evidence has linked the presence of *Aspergillus flavus* in residences and the presence of *Aspergillus flavus* precipitins in patient serum to neoplastic diseases and leukemia (Aleksandrowicz and Smyk, 1973; Dobrowolski and Smyk, 1993; Wray et al., 1982). To begin to address the concern regarding aflatoxin exposures in the indoor environment, we evaluated aflatoxin production by *Aspergillus flavus* on wet wallboard.

## MATERIALS AND METHODS

### Fungal Strains:

Two strains of *Aspergillus flavus*, both reported to produce aflatoxins, were obtained from American Type Culture Collection (ATCC, Rockville, MD) as lyophilized cultures. Strain ATCC 15547 was isolated from soil and produces aflatoxin B<sub>1</sub>. Strain ATCC 16875 was isolated from raw Spanish peanuts and produces aflatoxins B<sub>1</sub> and B<sub>2</sub>. Each culture was reconstituted in Sabouraud Dextrose broth (Difco, Detroit, MI) and incubated for four days at 28°C in a New Brunswick shaker at 60 RPM. Cultures were stored at 4°C. Each reconstituted strain was subcultured onto Czapek solution agar (Difco, Detroit, MI) plates to verify purity and identity of cultures.

### Preparation of Inocula:

Initially, inocula were prepared from potato dextrose agar culture plates (Remel, Lenexa, KS) and Sabouraud dextrose agar culture plates (Difco, Detroit, MI). In subsequent experiments, V-8 juice agar (Atlas and Parks, 1993) was used to increase spore production and decrease background aflatoxin levels in the inoculum. Each strain of *A. flavus* was grown on Sabouraud dextrose agar (SDA) or V-8 juice agar plates from 5 to 14 days at 28°C. Spores, mycelia and sclerotia were scraped from the SDA plate surface and suspended in sterile distilled water. Spores were harvested from V-8 juice agar plates by inverting culture plates over a funnel and shaking. Spores were suspended in 0.02% Tween 20. The concentration of culturable units in each inoculum was measured by culturing serial dilutions onto SDA plates. Aflatoxin content of each inoculum was measured as described below.

### Inoculation and Incubation Protocol:

Three common types of indoor building materials were tested: (1) plain wallboard (also known as gypsum/plasterboard) consisting of hemihydrated calcium sulfate sandwiched between cardboard; (2) wallboard with vinyl wallpaper affixed with cellulose-based wallpaper paste; and (3) wallboard with vinyl wallpaper affixed with wheat-based wallpaper paste. Since sterilization may change the nature of the substrate and the materials were new, samples were not sterilized. In initial experiments, approximately 20 gram samples of each type of tested substrate material were inoculated by pipetting 5 ml of inoculum over the surface of each dry sample. In subsequent experiments, wallboard pieces were prewetted (approximately 14 g sample and 10 g water). Each sample piece was then placed in a separate closed container with a sterile water reservoir to maintain high relative humidity and incubated at room temperature (23°C ± 1°C) or 28°C for up to 16 weeks, under fluorescent room lighting (on in the day and off at night and weekends) or in complete darkness.

Growth of *A. flavus* on randomly selected samples was verified by microscopic analysis of acid fuchsin-stained mounts (0.1% acid fuchsin in lactic acid) at 200X. Swabs of the surface and edges of wallboard samples were streaked onto Czapek solution agar plates for confirmation of *A. flavus* growth and continued aflatoxin production capability.

**Extraction and Assay Methods:**

The whole sample (substrate, mycelia, and spores) to be tested for aflatoxin concentration was pulverized in a Waring blender. The sample was extracted in 60% methanol in a ratio of 1 gram sample mass to 5 milliliter 60% methanol for one minute. Aflatoxin concentrations were measured with the Aflatest Mycotoxin Testing System (VICAM, Watertown, MA). In this system, highly specific immunoaffinity columns separate aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> from the crude extract. Aflatoxin concentrations (in ppb) were quantified by fluorometry (aflatoxins fluoresce naturally under ultraviolet light). The reported range of detectable aflatoxin concentrations using this method is 0-60 ppb in a 10 ml crude extract sample. (VICAM, 1994) When sample concentrations exceeded this range, smaller aliquots were tested and concentrations were extrapolated. Both inoculated and control samples of each wallboard type were analyzed. The results are reported as the measured value minus the original amount of aflatoxins in the inoculum minus background levels (background fluorescence) measured in extracts of uninoculated samples. The VICAM system protocol was validated by processing wallboard samples spiked with known amounts of a mixture of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> (Sigma, St. Louis, MO) dissolved in dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ).

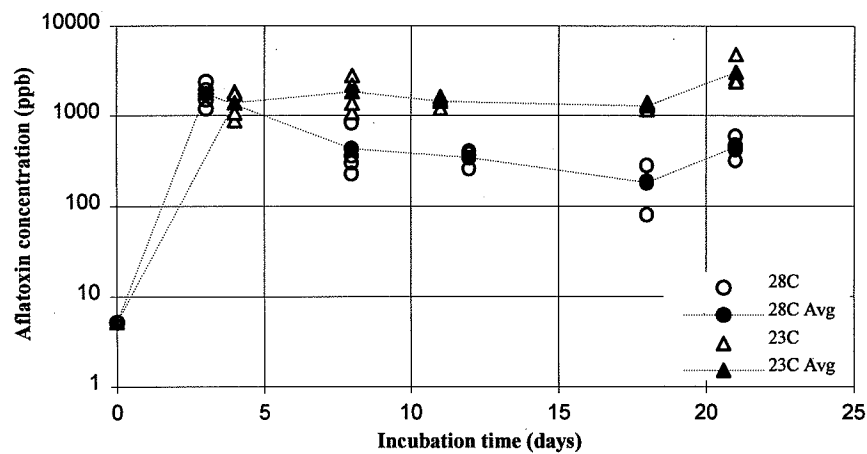
For chemical confirmation of aflatoxin presence, analysis by high performance liquid chromatography (HPLC) (Hewlett-Packard, Wilmington, DE) with a diode array detector, C-18 reverse phase column, and ethanol/water solvent, was performed on positive controls derived from Czapek solution agar plate cultures after processing through VICAM system.

**RESULTS**

**Aflatoxin Production on Culture Media:**

Aflatoxin production by each strain was verified over a span of 21 days with measurements at 3-4 day intervals. The highest aflatoxin production for ATCC strains 15547 and 16875 was observed when potato flake agar and potato dextrose agar (Remel, Lenexa, KS) were used, respectively. Strain ATCC 15547 was inconsistent in aflatoxin production on culture media. Detectable levels of aflatoxins were produced only during an initial survey of solid culture media, and not in any subsequent experiments. Levels of aflatoxin production by strain ATCC 16875 on potato dextrose ranged from a few hundred to a few thousand ppb and were consistently higher at 28°C than at room temperature (Figure 1).

Figure 1: Aflatoxin production by ATCC 16875 on potato dextrose agar

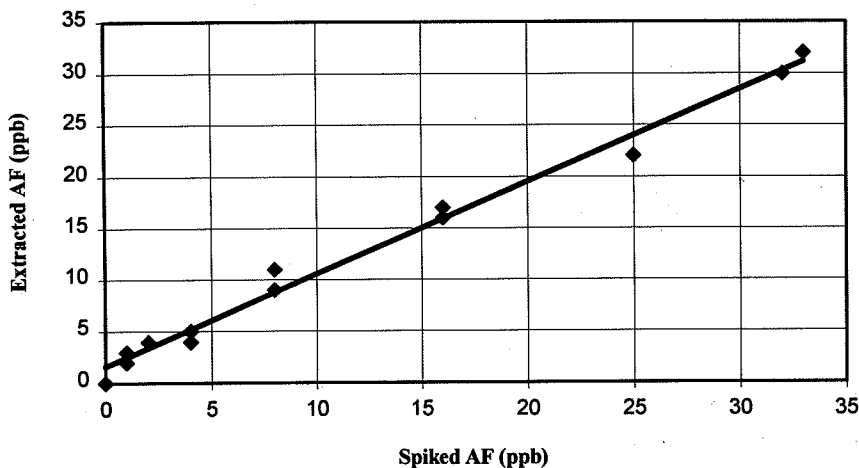


**Validation of VICAM System on Wallboard Samples:**

The VICAM extraction and analytical system were validated on cellulose wallpapered wallboard.

Univariate regression analysis of the concentration of aflatoxins spiked and recovered resulted in a slope of 0.90 and an R<sup>2</sup> of 0.99 (Figure 2).

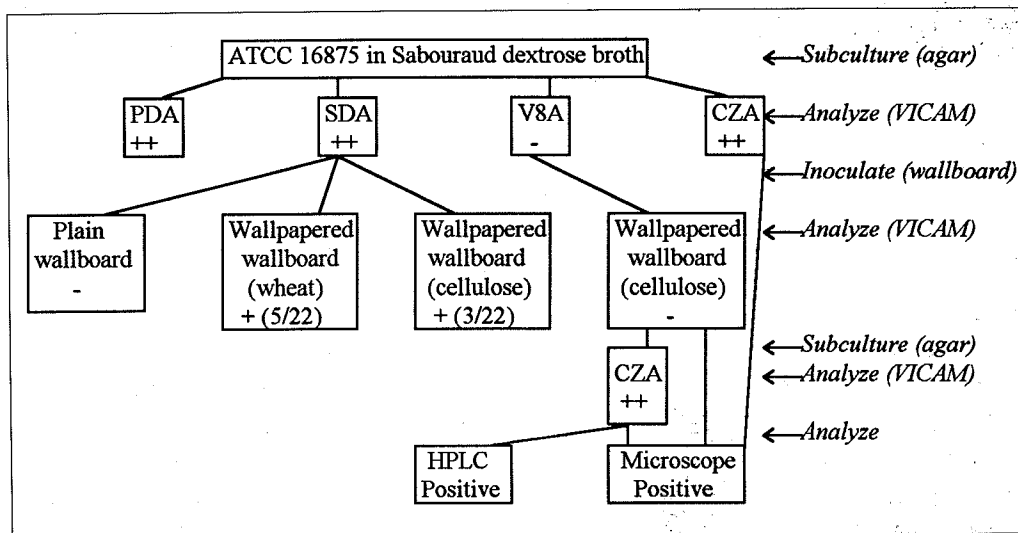
Figure 2: Aflatoxin concentration from aflatoxin-spiked cellulose wallpapered wallboard



**Aflatoxin Production on Wallboard:**

The overall protocol for the wallboard experiments is diagrammed in Figure 3.

Figure 3: Aflatoxin detection results on different growth media



PDA = potato dextrose agar; SDA = Sabouraud dextrose agar; V8A = V-8 juice agar; CZA = Czapek agar; HPLC = high performance liquid chromatography; Microscope = morphological identification under 200X; + = 2-12 ppb aflatoxin; ++ = >50 ppb aflatoxin; - = 0 ppb aflatoxin

Initially, we used all three types of wallboard and inocula produced on SDA. Three different inocula were prepared for each strain from SDA plates, with initial aflatoxin levels ranging from <5 ppb to 550 ppb and colony-forming unit (CFU) concentration counts ranging from  $1.85 \times 10^5$  CFU/ml to  $4 \times 10^6$  CFU/ml. No detectable aflatoxins were produced on plain (unpapered) wallboard samples by either strain and ATCC 15547 did not produce measurable levels of aflatoxin on any wallpapered wallboard samples. When ATCC 16875 inocula were prepared from SDA, aflatoxins were detected in 5/22 wallpapered (cellulose pasted) wallboard samples and 3/22 wallpapered (wheat pasted) wallboard samples and only in samples where inocula contained <60 ppb aflatoxins. Levels in positive samples ranged from 2-12 ppb with an average of 5.7 ppb which is marginally above the variability of the analysis method.

Considering that the presence of variable amounts of aflatoxin in inocula might have influenced these results, we used V-8 juice agar-derived inocula that contained no detectable aflatoxin. The inoculum prepared from ATCC 16875 grown on V-8 juice agar had below minimum detection level of aflatoxins (<5 ppb) and approximately  $3.2 \times 10^4$  CFU/ml. In these experiments, there were no differences between aflatoxin levels measured from controls and inoculated samples that were supporting visible growth, even after 16 weeks of incubation. *A. flavus* was re-isolated from inoculated samples (but not from controls) and produced up to 730 ppb aflatoxin on Czapek solution agar.

## DISCUSSION

Aflatoxin production by *Aspergillus flavus* is conditional and dependent upon many factors (Bennett et al., 1978; Davis et al, 1966; Joffe and Lisker, 1969). Only some *Aspergillus flavus* strains are genetically capable of producing aflatoxin (designated as toxigenic). Among these strains, external factors play a significant role in the expression of the gene for aflatoxin production (Bennett et al., 1978; Bennett & Deutsch, 1985; Bu'lock, 1980; Davis et al., 1966; Joffe & Lisker, 1969). A combination of environmental factors, such as the amount and wavelengths of available light, pH, growth substrate, relative humidity, temperature, substrate and

the presence or absence of other microorganisms may affect the ability of a toxigenic strain to produce aflatoxin (Bean & MacFall, 1982; Joffe and Lisker, 1969; Northolt & Bullerman, 1982).

Our studies confirmed the ability of two known toxigenic strains of *Aspergillus flavus* to produce detectable amounts of aflatoxin when grown on certain culture media. Incubation time and temperature affected the levels of aflatoxin detected (Figure 1) and there were large variations in aflatoxin recoveries within the incubation time periods even though replicate samples were identically inoculated, incubated and analyzed. At concentrations above the range of the system (i.e., >60 ppb), extrapolation errors could have occurred, but are unlikely to explain the large variability among replicate samples. It is possible that variable degradation of aflatoxin in the inoculum and concurrent variable aflatoxin production by the fungus contributed to variability among replicates. It could also be that aflatoxin production is inherently variable and unpredictable under these conditions (Mayne et al., 1971; Wei & Jong, 1986).

Although both strains of *A. flavus* produced visible growth on wallboard, one (ATCC 15547) produced no aflatoxin and the other (ATCC 16875) produced very little. There have been previous difficulties in obtaining consistent results with strain ATCC 15547 (Wei & Jong, 1986). The few marginally positive results obtained with ATCC 16875 were likely due to method variability and extrapolation error rather than actual increases in pre-incubation aflatoxin levels. This is confirmed by the lack of positive results in a second set of more carefully controlled experiments.

In the initial series of experiments with ATCC 16875, detection of aflatoxin above background appeared to depend on the initial concentration of aflatoxin in the inoculum; only the lower concentration inocula resulted in final aflatoxin concentrations above background. In fact, aflatoxin recovery from most samples was much less than levels in the initial inoculum. Toxin-producing strains of *A. flavus* have been shown to degrade aflatoxin (Doyle & Marth, 1956) and this kind of breakdown may have driven our results. It is also possible that the presence of aflatoxin in the original SDA inocula prevented the production of additional toxin. However, the absence of aflatoxin production when V-8

juice agar inocula (where aflatoxin concentration in the inoculum was less than the detection limit of the assay) were used argues against this hypothesis.

Although we attempted to simulate an indoor environment, our laboratory conditions (i.e., high relative humidity, constant saturation of substrate, and constant temperature) were not truly realistic. In the field, additional environmental stresses, such as repeated moist/dry cycles, temperature fluctuations, competition with or stimulation by other microorganisms and nutrient deprivation, often occur, all of which could affect aflatoxin production (Faraj, 1991). These environmental stressors can also control the composition of fungal populations in a given environment, and may select or stimulate strains that can produce toxin in the given environment. Our studies apply only to the strains tested. Confirmation that *A. flavus* does not produce aflatoxins on wallboard will require further study using environmentally isolated strains and analyzing field collections specifically for aflatoxins where *A. flavus* is present. In addition, other potentially toxic metabolites may have been produced by our tested strains that would not have been detected by the Aflatest Mycotoxin Testing System.

We have demonstrated that *A. flavus*, strain ATCC 16875, does not produce aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> when growing on either plain or wallpapered wallboard under our environmental conditions even though the fungus remains able to produce toxin on culture media. It is clear from our data that detection of a toxigenic fungal species in an indoor (or even outdoor) environmental sample does not necessarily imply that mycotoxins are also present. Actual measurement of specific toxins, or toxicity in a more general assay, is essential to document toxin-specific health risks. However, in view of the many different toxins and allergens known to be produced by fungi under some conditions, conservative measures (i.e., personal protective equipment) should always be used when handling materials supporting fungal growth.

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