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Mycotoxigenic isolates and toxin production on buckwheat and rice hulls used as bedding materials

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SUMMARY

A pre-evaluation of the samples of both buckwheat and rice hulls, planned for use as pillow fill-materials, showed the presence of *Aspergillus flavus, A glaucus,* and *Penicillium* spp. Buckwheat- and rice-hull media (BHM and RHM) inoculated with *A. flavus* both supported the production of aflatoxins (AFB_1 and AFG_1) in the parts per million (ppm) range; BHM yielded approximately twice the quantity of both AFB_1 and AFG_1 than did RHM. Both BHM and RHM inoculated with *Fusarium tricinctum* yielded trichothecenes (T-2 toxins) in the ppm range, with the BHM producing approximately three times more T-2 toxins than the RHM. Also, *F. tricinctum* grown on both media produced several metabolites which included HT-2, 3′-OH T-2, neosolariol, T-2 triol, and T-2 tetraol. The BHM yielded all of the above, while the RHM failed to support the production of the 3′-OH T-2 toxin. In addition, neither medium inoculated with *Myrothecium roridum* yielded any detectable levels of macrocyclic trichothecenes. The results indicated that these materials have the potential to become contaminated with mycotoxins.

INTRODUCTION

Aflatoxins, secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus* [3,5], have been shown to be hepatocarcinogenic, mutagenic, teratogenic, and toxic. Macrocyclic trichothecenes, metabolites of the fungus *Myrothecium roridum,* and sometimes *M. verrucaria,* have been reported to function as plant-root pathogens [6], and are known to be produced by *M. roridum* isolated from muskmelons, *Cucumis melo* [1]. The presence of trichothecenes also has been observed in seedgrain [16].

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Rice hulls contaminated with bacteria (*Corynebacterium equi*) were reported to cause purulent pneumonia in some Venezuelan thoroughbred horses [17]. Rice-husk dust has been implicated as the causative agent of 'Rice Millers' Syndrome' in Malaysian rice millers; symptoms include acute and chronic irritant effects of the eyes, skin, and upper respiratory tract. It has been suggested that this dust causes allergic responses such as nasal catarrh, tightness of the chest, asthma, and eosinophilia in exposed persons. Radiological opacities in the chest, possibly representing early silicosis or extrinsic allergic alveolitis, also have been reported to be associated with rice-husk dust [7]. In addition, *A. parasiticus* has been shown to produce aflatoxicol, as well as aflatoxins, in both moldy corn and buckwheat flour [11]. Interestingly, aflatoxin production by *A. flavus* in rice seed has been reported to be less extensive in unhulled rice, as compared to hulled rice [9].

In the study reported herein, the natural mycotoxigenic fungal flora were determined in buckwheat hulls and rice hulls. In addition, rice-hull (RHM) and buckwheat-hull media (BHM) were inoculated with *A. flavus*, *Fusarium tricinctum* and *M. roridum* to determine the potential of these substrates for the production of aflatoxins and/or trichothecenes.

MATERIALS AND METHODS

Buckwheat and rice hulls

Dried buckwheat hulls (3000 g) used in this study were provided in a sealed plastic bag from a local bedding manufacturer in Richmond, VA, and the dried rice hulls (1000 g) were provided by an out-of-state processor, also in a sealed plastic bag. Several aliquots of both the buckwheat and rice hulls were removed (via sterile procedures) and subjected to microbial analysis. The remaining portions of these samples were used as media substrates in both the mold-growth and toxin-production sections of this study.

Mycotoxigenic fungal survey

Both surface-disinfected and nondisinfected buckwheat and rice hulls were evaluated (prior to initiation of this study) as to their natural fungal contamination. Surface-disinfected hulls were treated by standard methods using sodium hypochlorite (NaOCl), whereas nondisinfected hulls of both substrates were not subjected to this treatment. Naturally occurring fungal flora were determined after 14 days at ambient temperatures.

Preparation of media for toxigenic mold growths

Sixty 500 ml prescription bottles stoppered with diSpo plugs (American Scientific Products, McGraw Park, IL) were used. Each bottle contained a 10 g aliquot of either ground buckwheat hulls or rice hulls with 40 ml of distilled water. The bottles were autoclaved at 15 psi for 20 min and allowed to cool prior to inoculation.

Inoculation procedures

Three different fungi were used to inoculate the BHM and RHM. *A. flavus* ATCC 15548 (maintained on potato-dextrose-yeast agar), a known aflatoxin producer, was provided by Virginia Commonwealth University, Richmond, VA, originally from G.C.L. *F. tricinctum* NRRL 3299 cultures (known to produce tricothecenes) were provided by W.M.H. *F. tricinctum* was maintained on potato-dextrose agar (PDA) slants prior to inoculation. In addition, *M. roridum* MRT-100 (a macrocyclic trichothecene producer), also maintained on PDA slants, was provided by G.A.B. Equivalent quantities of *A. flavus* conidia from a single spore culture were used to inoculate the BHM and RHM. Equivalent quantities of *F. tricinctum* and *M. roridum* conidia also were utilized as inoculants.

After inoculation, the bottles were shaken to evenly disperse the hulls and fungus. All cultures were maintained in darkness at 20 ± 2°C for a total of 51 days, prior to analysis.

Extraction of cultures

All 10 g buckwheat- and rice-hull cultures were extracted with 200 ml, then 100 ml, of ethyl acetate, and the extracts were combined and evaporated.

Residues of the extracts were quantitatively transferred with acetone for purification and analysis [12].

Aflatoxin analysis

Extracts were assayed quantitatively for aflatoxins by thin-layer chromatography (TLC) with appropriate standards [19]. A chloroform/ethyl ether/acetic acid (170:30:10, v/v) solvent system was used to develop the plates which were read by spot meter densitometry [4].

Trichothecene analysis

The ethyl acetate extracts were initially analyzed by TLC, but extensive interferences were encountered. Extracts were then dissolved in acetonitrile and defatted with petroleum ether. Acetonitrile was evaporated and extracts were purified by silica column chromatography [12] for gas-liquid chromatographic analysis (GLC) with electron capture detection of heptafluorobutyryl derivatives by the method of Scott et al. [14]. Trichothecenes (T-2 toxins) in extracts were confirmed by mass spectrometry (GLC/MS) as described by Voyksner et al. [18]. T-2 toxin was quantified by GLC, but T-2 toxin metabolites were identified qualitatively by GLC/MS.

Macrocyclic trichothecene toxin isolation and analysis

Analysis for macrocyclic trichothecenes (roridins and verrucarins) followed standard procedures as outlined by Bean et al. [1]. Detection limits in these procedures were ≥ 1 ppb.

RESULTS AND DISCUSSION

Fungi isolated from disinfected and nondisinfected buckwheat and rice hulls

Table 1 lists the fungi isolated from disinfected and nondisinfected buckwheat and rice hulls. From these data, it is evident that of the fungi observed in this study, only *A. flavus* occurred on both substrates (buckwheat 7% and rice 1%). Based on the test results from the control samples, the presence of *A. flavus* did indicate that there was a potential for aflatoxin occurrence, although there was no aflatoxin present at detectable levels. It has not been determined whether this isolate of *A. flavus* is capable of producing aflatoxins under laboratory conditions.

Aflatoxin (AF) production by A. flavus ATCC 15548

AFB_1 and AFG_1 were produced by the *A. flavus* inoculum on both BHM and RHM. BHM appeared to be a better substrate for the production of aflatoxins than RHM (Table 2). Analysis of the BHM showed a 134% higher quantity of AFB_1 production compared to the AFB_1 production in RHM. Similarly, analysis of the BHM indicated a 94% higher quantity of AFG_1 production compared to RHM. Also, *A. flavus* grown on either medium produced more AFG_1 than AFB_1 (BHM = 39% more AFG_1 than AFB_1; RHM = 68% more AFG_1 than AFB_1). In all extracts analyzed, AFG_1 production was greater than that of AFB_1. In addition, no aflatoxins were found in the control extract samples.

These results indicate clearly that BHM is a better substrate for the production of both AFB_1 and AFG_1 than the RHM. Substantially more aflatoxins were produced on BHM, implying that a more rapid and extensive contamination of this substrate occurred under these experimental conditions. It also appears that the isolate of *A. flavus* produced more AFG_1 than AFB_1 on both media.

T-2 toxin production by F. tricinctum

Table 2 indicates that the BHM produced nearly three times more T-2 toxin than the RHM. Apparently, the BHM provides a better mycotoxin-producing substrate than the RHM, since these values are comparable to the observed aflatoxin levels on each substrate.

Toxin production by M. roridum

Assay results indicated that neither roridins nor verrucarins were present in either BHM or RHM media inoculated with *M. roridum*. It appears that under these experimental conditions and detection

Table 1

Fungi isolated from disinfected and nondisinfected buckwheat and rice hulls

Fungi	Substrate			
	buckwheat		rice	
	disinfected	nondisinfected	disinfected	nondisinfected
A. glaucus	72[a]	73	–	97
A. flavus	–	7	–	1
A. ochraceus	–	1	–	–
Penicillium spp.	–	19	–	2

[a] Percentage of 200 hulls.

limits neither of the tested media has the potential for allowing production of detectable quantities of cyclic trichothecenes by an isolate of *M. roridum* known to be toxigenic under laboratory conditions.

Metabolite production

Table 3 lists specific metabolites produced on BHM and RHM substrates inoculated with *F. tricinctum*. These metabolites were confirmed by GLC/MS, but not quantified with respect to concentration.

General discussion

With regard to aflatoxin production by *A. flavus* ATCC 15548 in this study, we found that BHM averaged approximately 14 μg/g of AFB_1 and RHM averaged approximately 6 μg/g of AFB_1. The AFG_1 levels were approximately 19 μg/g and 10 μg/g for these two media, respectively. When these values were compared with production levels on other substrates, we found that both rice and buckwheat could be classified as low-to-moderate producers of AFB_1 [8]. For example, on inoculated

Table 2

Aflatoxin and trichothecene production (ppm) on buckwheat and rice hulls inoculated with *A. flavus* ATCC 15548, *F. tricinctum* NRRL 3299, and *M. roridum* MRT-100

No aflatoxins or trichothecenes other than those shown in the table were detected in any samples (including controls).

Inoculum	Substrate			
	buckwheat		rice	
	AFB_1	AFG_1	AFB_1	AFG_1
A. flavus	13.8 ± 2.6	19.2 ± 2.9	5.9 ± 1.3	9.9 ± 2.1
	T-2 toxin		T-2 toxin	
F. tricinctum	18.9 ± 5.3		6.5 ± 0.9	
M. roridum	n.d.[a]		n.d.	

[a] n.d. = none detected (trichothecenes).

Table 3

Metabolite production on buckwheat and rice samples inoculated with *F. tricinctum* using GLC/MS

Substrate	Metabolite
Buckwheat	T-2 toxin
	HT-2
	3'-OH T-2
	Neosolaniol
	T-2 triol
	T-2 tetraol
Rice	T-2 toxin
	HT-2
	Neosolaniol
	T-2 triol
	T-2 tetraol

buckwheat and rice hulls, more AFB_1 was produced than on ground celery seeds or rosemary leaves, and about the same quantity as found on ground ginger. BHM and RHM yields were certainly not in the range of 136 μg/g of AFB_1, found in ground sesame seeds, or in cultures of inoculated York soybeans where this same isolate of *A. flavus* produced over 1500 μg/g of AFB_1 [15]. Blackberries were shown to yield about 7.5 μg/g AFB_1, cherries averaged 2 μg/g, and russet potatoes produced approximately 1 μg/g of AFB_1, while strawberries and mushrooms (*Agaricus bisporus*) average <1 μg/g [8]. Based on studies with a single isolate of *A. flavus*, it appears that on BHM and RHM *A. flavus* is a low-to-moderate producer of AFB_1.

A comparison of the trichothecene levels produced on these substrates in this study also can be made. For example, Mirocha et al. [10] found corn to contain approximately 0.08 μg/g T-2 toxin, and they found mixed feed to contain 0.5 μg/g T-2 toxin. In addition, Puls and Greenway [13] reported a concentration of 25 μg/g T-2 toxin in barley. In comparison with these data, both BHM and RHM support potentially high trichothecene production levels.

Myrothecium toxin levels, if any, were below the detectable range of ≥ 1 ppb. This may have been the result of problems associated with growth of the strain or in the media themselves. We feel that it is more likely a media preference of the fungi, since this culture has previously produced cyclic trichothecenes successfully on a variety of other substrates. Apparently, under these conditions BHM and RHM do not allow for cyclic trichothecene production.

We must also consider that *Aspergillus* spp. and other fungi were detected on the external surface of the substrates used in these studies. In addition, *A. flavus* also was found to be present internally in surface-disinfected hulls. When one considers these results plus the low-to-moderate production levels of AFB_1 and the high levels of T-2 toxin, it appears that these materials could become a potential health hazard. Under proper conditions of moisture, mold contamination, and temperature, toxigenic fungi could grow and produce toxins. This risk is much more substantial for these types of materials than some other standard types of bedding materials from both natural and synthetic sources (i.e., cotton, man-made materials). Numerous states have bedding and upholstered furniture laws which limit and control certain fill materials. Many conditions simply may require the labeling of specific fill materials and other contents [2]. With buckwheat and rice hulls used as fillers, we feel certain that there should be a label indicating the contents, especially if the buckwheat hulls and rice hulls are not sterilized or proven to be spore- and/or mold-free when manufacturing begins. A warning label indicating disposal after severe water-soaking exposures is necessary and would be appropriate.

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