Natural Occurrence of Fumonisin in Rice with Fusarium Sheath Rot Disease

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ABSTRACT

Abbas, H. K., Cartwright, R. D., Shier, W. T., Abouzied, M. M., Bird, C. B., Rice, L. G., Ross, P. F., Sciumbato, G. L., and Meredith, F. I. 1998. Natural occurrence of fumonisins in rice with Fusarium sheath rot disease. Plant Dis. 82:22-25.

Twenty samples of rough rice (*Oryza sativa*) (unpolished kernels) collected during the 1995 harvest season from Arkansas (seven samples) and Texas (13 samples) were obtained from rice fields known to include plants with symptoms of Fusarium sheath rot putatively caused by *Fusarium proliferatum*. Samples were analyzed for fumonisin B₁ (FB₁) at three laboratories using three different extracting solvents by high-performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA) methods. Forty percent of the samples were positive for FB₁ at levels ≤ 4.3 $\mu\text{g/g}$ by HPLC. The same samples contained FB₁ at ≤ 3.6 $\mu\text{g/g}$ when measured by an ELISA method. Most samples that were positive for FB₁ were positive for fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) by HPLC at levels ≤ 1.2 $\mu\text{g/g}$. Very good agreement was obtained among the two laboratories using HPLC methods and the third using ELISA. Shelling of the unpolished rice results in hull and brown rice fractions. In a sample that contained 4.3 $\mu\text{g/g}$ in whole kernels, the fumonisin level was very high in hulls (≤ 16.8 $\mu\text{g/g}$) and low in brown rice (≤ 0.9 $\mu\text{g/g}$). Milling of brown rice results in bran and white rice fractions. Fumonisin was found in bran at a level of ≤ 3.7 $\mu\text{g/g}$ but were below the level of detection by HPLC in white rice. The presence of fumonisins (FB₁, FB₂, and FB₃) was confirmed by fast atom bombardment/mass spectrometry. This is the first report of fumonisins in naturally contaminated rice in the United States.

Additional keyword: mycotoxins

Fumonisin is a series of structurally related sphingosine analog toxins (29,37) produced by *Fusarium moniliforme* and other fungi (5,16,18,22,27,30,33). The most abundant and one of the most active members of this series is fumonisin B₁ (FB₁) (1,2,35). Since their discovery in 1988 (16) and the first reports of natural occurrence in corn in 1989 (36) and 1990 (28,32), the fumonisins have rapidly become an important class of mycotoxins. Their natural occurrence in corn has been reported in many geographical areas around the world (9,17,19,24,26,31,32,34). Recently, FB₁ was reported in syrup made from sorghum (14).

The fungi that produce fumonisins are well-known pathogens of a variety of plants (8,11,21,23). During the last 3 years, Fusarium sheath rot has been reported as a disease in the southern United States, where rice is a major crop (11–13). Two

cultivars (Bengal and Cypress) of rice are very susceptible to this disease (11,13) caused by *F. proliferatum* (11). *F. proliferatum* produces mycotoxins characteristic of *Fusarium* spp., including fumonisin, moniliformin, and beauvericin (25). Health concerns about FB₁ and other fumonisins in corn has led investigators to quantify their presence in products destined for human or animal use. However, to date, fumonisins have not been reported in rice. We report here detection of fumonisins by high-performance liquid chromatography (HPLC) and competitive direct enzyme-linked immunosorbent assay (CD-ELISA) in rough rice kernels harvested from fields known to include plants with Fusarium sheath rot. We also investigated the levels of fumonisins in various rice fractions produced by shelling and milling.

MATERIALS AND METHODS

Rice samples. Twenty samples of combine-harvested rough rice were collected during the 1995 harvest of commercial rice fields in Arkansas and Texas. Samples of ≥ 0.5 kg were collected randomly from each field and used for fungal isolation and chemical analysis. Samples were transferred to polyethylene bags and kept at -20°C until analyzed. The number of sam-

ples, the cultivar sources, and the collection sites are listed in Table 1.

Disease symptoms. Symptoms of Fusarium sheath rot of rice are as previously described (11,12) and include: (i) blanked or partially blanked panicle with reddish brown to off-white florets or kernels often covered with a white to pinkish white powder consisting of microconidia and conidiophores of *F. proliferatum*; (ii) the flag leaf sheath develops a rapidly enlarging lesion, first dull to dark brown and later off-white to tan with a reddish brown border, that eventually encompasses the entire sheath and may result in death of the leaf blade; (iii) lower leaf sheaths may eventually develop lesions as well, but rarely more than two leaf sheaths show symptoms; and (iv) a dense white to pinkish white powder consisting of microconidia and conidiophores of *F. proliferatum* covers the sheath lesions, especially evident during humid periods.

Fungal isolation. Isolates of *F. proliferatum* were recovered from rice tissue showing characteristic Fusarium sheath rot symptoms using standard methods (3,20). The percentage of symptomatic plants was determined by random grid (quadrant) sampling at 50 locations within each field. A plastic square (625 cm^2) was thrown at random 50 times in each affected field, and symptomatic and nonsymptomatic tillers within the square were counted. The number of tillers with symptoms was divided by the total number of tillers and multiplied by 100 to determine percent incidence. Fields were sampled once. *Fusarium* spp. were identified using published methods (21,23). *F. proliferatum* used in tests were grown from a single spore and maintained on silica gel at 4°C , as described by Windels et al. (38).

Table 1. Rough rice samples, sample numbers, and collection sites used in this study^a

Cultivar	Sample numbers	Collection site
Bengal	1, 4, 18	Arkansas
Cypress	7, 8, 10, 12	Arkansas
	3, 5, 6, 9, 16, 17, 19, 20	Texas
Gulfmont	13, 15	Texas
Lamont	14	Arkansas
	2, 11	Texas

^a Sample size ≥ 500 g each.

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Accepted for publication 22 September 1997.

Publication no. D-1997-1118-03R
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Fumonisin analysis. Each sample was ground to the consistency of powder using a grinder (Omni-Mixer, Sorvall, Inc., Newtown, CT) and divided into subsamples of 25 or 50 g each. Three laboratories were involved in the testing of these samples for fumonisins:

Laboratory 1 was the Southern Weed Science Laboratory (SWSL) in collaboration with the University of Minnesota. Samples were analyzed for fumonisins by HPLC according to the method of Shephard et al. (28). Briefly, samples were extracted with methanol:water (1:1, vol/vol), centrifuged, and filtered. The filtrate was applied to a Bond-Elut SAX cartridge and eluted with 0.5% acetic acid in methanol. The solution was evaporated, and the residue was derivatized with *o*-phthalaldehyde (OPA) and injected onto the HPLC column. The limit of detection was 0.5 µg/g.

Laboratory 2 was USDA-APHIS, NVSL, Ames, Iowa. This laboratory used the method previously described in detail (26). Briefly, samples were extracted with 50% acetonitrile in water (vol/vol) by shaking for 30 min and filtered. The filtrate was purified on C₁₈ solid-phase extraction columns, and the eluate was derivatized with OPA and injected onto the liquid chromatograph column. Limit of detection was 0.5 µg/g.

Laboratory 3 was Neogen Corporation. Samples were extracted with 70% (vol/vol) methanol:water and filtered. CD-ELISA

was used to determine total fumonisins. This procedure has been described in detail by Abouzied et al. (6) and has a limit of detection of 0.1 µg/g.

Identity confirmation. The identity of toxins present in harvested rice samples was confirmed by fast atom bombardment/mass spectrometry (FAB/MS) (5) or continuous flow/fast atom bombardment/mass spectrometry (CF/FAB/MS) (18) at the University of Minnesota, St. Paul. High resolution fast atom bombardment/mass spectrometry (HR FAB/MS) was performed to confirm the elemental formula of each toxin in rice samples 3, 6, 16, and 17, at the U.S. Food and Drug Administration, Washington, DC.

Distribution of FB₁ in shelling and milling fractions. One kg of the unpolished rice sample 3-2 was used for this study. Shelling and milling of unpolished rice was performed at the quality control laboratory at Uncle Ben's Rice Company (Greenville, MS) using the standard commercial procedure. In the shelling process, the rice sample was fractionated into brown rice and hulls. In the milling process, bran was separated from brown rice, leaving the white rice fraction.

RESULTS

Fumonisin B₁ was found in readily detectable levels (>1 µg/g) in six out of 20 (30%) independent rice samples (Table 2). As determined by HPLC at SWSL and the University of Minnesota (lab 1), the six

positive samples had levels of FB₁ ranging from 3.1 to 4.3 µg/g. Including FB₂ and FB₃, total fumonisins ranged from 3.6 to 6.6 µg/g in the same samples. The same six samples independently assayed by HPLC at NVSL in Ames (lab 2) contained FB₁ at 2.3 to 4.0 µg/g, while total fumonisins ranged from 2.3 to 5.4 µg/g.

The presence of total fumonisins in these samples correlated well with percent sheath and panicle symptoms ($r = 0.97$) and with the percent recovery of *F. proliferatum* from grain obtained from fields infected with Fusarium sheath rot ($r = 0.97$) (Table 3). Similarly, percent sheath and panicle symptoms correlated well with percent recovery of *F. proliferatum* from grain ($r = 0.99$).

FB₁, FB₂, and FB₃ were present, as shown by both FAB/MS and CF/FAB/MS. The elemental formula of each was confirmed by FAB/MS. FB₁ levels as determined by CD-ELISA were similar to but slightly lower than HPLC levels. Measurements by the two HPLC methods were highly correlated for FB₁ ($r = 0.97$), FB₂ ($r = 0.94$), and FB₃ ($r = 0.64$). The CD-ELISA measurements were well correlated with the results of HPLC measurements from both laboratory 1 ($r = 0.95$) and laboratory 2 ($r = 0.95$). FB₂ and FB₃ could not be measured separately by CD-ELISA because this antibody reacts with all fumonisins to some extent.

One sample (no. 19) was used to determine the fate of fumonisin in the shelling and milling processes. The shelling process divides the unpolished rice into hulls and brown rice, while milling gives fractions of bran and white rice. Shelling substantially reduced FB₁ levels in the brown rice fraction by 75 to 80%, from 4.7, 2.4, and 3.5

Table 2. Fumonisin levels in unpolished rice samples

Sample no. ^a	Laboratory no. ^b	Assay method ^c	Fumonisin level (µg/g)			Total (µg/g)
			FB ₁	FB ₂	FB ₃	
3	1	HPLC	3.5	1.2	0.5	5.2
	2	HPLC	3.0	1.1	ND	4.1
	3	CD-ELISA				3.3
19	1	HPLC	4.3	1.0	0.5	5.8
	2	HPLC	3.3	0.9	0.5	4.7
	3	CD-ELISA				3.1
4	1	HPLC	Trace ^d	ND	ND	Trace
	2	HPLC	0.5	ND	ND	0.5
	3	CD-ELISA				Trace
6	1	HPLC	3.1	0.5	ND	3.6
	2	HPLC	2.3	ND	ND	2.3
	3	CD-ELISA				1.9
20	1	HPLC	3.1	0.8	0.5	4.4
	2	HPLC	2.9	0.7	ND	3.6
	3	CD-ELISA				3.0
9	1	HPLC	0.9	ND	ND	0.9
	2	HPLC	Trace	ND	ND	Trace
	3	CD-ELISA				Trace
16	1	HPLC	4.1	1.0	0.5	5.6
	2	HPLC	4.0	0.9	0.5	5.4
	3	CD-ELISA				3.6
17	1	HPLC	3.3	0.9	0.6	4.8
	2	HPLC	3.1	0.9	0.5	4.5
	3	CD-ELISA				2.9

^a Fumonisin levels were not detectable (ND) in the other 12 of 20 samples at 0.5 µg/g when determined by high-performance liquid chromatography (HPLC) or at 1 µg/g when determined by competitive direct enzyme-linked immunosorbent assay (CD-ELISA) (Veratox).

^b Laboratory 1 included Southern Weed Science Laboratory and the University of Minnesota; laboratory 2 was NVSL, USDA-APHIS; laboratory 3 was Neogen Corp.

^c CD-ELISA data shown for total fumonisins because of cross-reactivity among fumonisins.

^d ≤0.3 µg/g.

Table 3. Incidence of Fusarium sheath rot symptom and percent recovery of *Fusarium proliferatum* from designated field and grain samples

Sample	Sheath and panicle symptoms ^a (%)	Recovery from grain ^b (%)
1	1	0
4	5	2
18	<1	0
7	1	1
8	3	5
10	<0.1	1
12	2.5	8
3	20 ^c	100
5	... ^d	6
6	20 ^c	100
9	3	12
16	20 ^c	100
17	20 ^c	100
19	20 ^c	100
20	20 ^c	100

^a Percentage of symptomatic plants was determined as described in Materials and Methods.

^b Based on two lots of 100 seeds plated on Nash-Snyder agar (20). Grain was collected from combine samples from each field.

^c Different areas of same field.

^d Field survey was not performed.

µg/g in the three laboratories to 0.9, 0.6, and 1.2 µg/g, respectively. Milling gave a white rice fraction with undetectable levels of fumonisins (Table 4). FB₂ and FB₃ were decreased in the shelling and milling processes to undetectable levels in both brown rice and white rice.

DISCUSSION

Fumonisin levels in food and feed crops are an important public health concern. The cause of most human cancer is not known (10), but FB₁ as a putative environmental tumor promoter (16) could contribute to it, especially if exposure is long-term. Fumonisin contamination of animal feed has caused illness and death of livestock, with associated economic losses (27). Guidance levels for fumonisin in animal feed issued by the American Association of Veterinary Laboratory Diagnosticians, Mycotoxin Committee, vary by species, but levels above 5 µg/g can be harmful to horses (15). Rough rice and rice hulls, which are sometimes fed to livestock, contained concentrations of fumonisins above 5 µg/g. White rice, the form of rice most widely consumed in human diets in this country, did not contain detectable levels. Therefore, the fumonisins are localized primarily in the hulls and bran. Nonetheless, this study demonstrates that rice and products derived from rice may potentially contain fumonisins and should be monitored for fumonisins to assure maximum food safety. Fumonisin is heat-stable and would likely not be destroyed by rice cooking methods (4,7). Additional studies are needed to determine the prevalence of fumonisins in rice grown in other geographical areas and in food and feed products derived from rice.

Fusarium sheath rot of rice is a recently reported problem in the United States, and

the disease itself remains poorly understood. While reproduction of symptoms under greenhouse conditions has been reported, reproduction of the disease under field conditions using various isolates of *F. proliferatum* has been largely unsuccessful, leading to speculation about conditions needed for infection and disease development. The fungus appears to be a weak pathogen under most conditions, and other factors may be involved in the field. Other conditions result in blanked panicles in U.S. rice fields, and all causes of these symptoms need to be determined and clarified. Nevertheless, the widespread *F. proliferatum* contamination of commercial rice fields growing the newly released cultivars is a cause for concern.

ACKNOWLEDGMENTS

We thank Bobbie J. Johnson, USDA-ARS, SWSL, Stoneville, MS, for her help in preparation of this manuscript. We thank C. J. Mirocha and W. Xie for confirming toxins by CF/FAB/MS and T. Krick for confirming toxins by FAB/MS, University of Minnesota, St. Paul; and Mary W. Trucksess and Michael E. Stack, U.S. Food and Drug Administration, Washington, DC., for confirming the presence of fumonisins. We also thank Dorothy B. Davis and David Weddington, Uncle Ben's Rice Company, Greenville, MS, for shelling and milling a rice sample to obtain various fractions. In addition, we thank C. M. Ocamb, USDA Forest Service, St. Paul, MN, and James Correll, Department of Plant Pathology, University of Arkansas, Fayetteville, for confirmation of the identity of *F. proliferatum*.

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Table 4. Fumonisin levels in rice fractions from field sample no. 19

Rice fraction	Laboratory no. ^a	Assay method	Fumonisin levels (µg/g) ^b			Total (µg/g)
			FB ₁	FB ₂	FB ₃	
Unpolished rice	1	HPLC	4.7	1.0	0.6	6.3
	2	HPLC	2.4	0.7	1.3	4.4
	3	CD-ELISA ^c				3.5
Hulls	1	HPLC	13.0	2.6	1.2	16.8
	2	HPLC	8.4	1.3	1.6	11.3
	3	CD-ELISA				14.5
Brown rice	1	HPLC	0.9	ND	ND	0.9
	2	HPLC	0.6	ND	ND	0.6
	3	CD-ELISA				1.2
Bran	1	HPLC	3.1	0.6	ND	3.7
	2	HPLC	1.7	0.5	0.5	2.7
	3	CD-ELISA				3.4
White rice	1	HPLC	ND	ND	ND	ND
	2	HPLC	ND	ND	ND	ND
	3	CD-ELISA				ND

^a Laboratory 1 included Southern Weed Science Laboratory and the University of Minnesota; laboratory 2 was NVSL, USDA-APHIS; laboratory 3 was Neogen Corp.

^b HPLC = high-performance liquid chromatography; CD-ELISA = competitive direct enzyme-linked immunosorbent assay. CD-ELISA data are given for total fumonisins because of cross-reactivity among fumonisins.

^c ND = no detectable fumonisin at 0.5 µg/g when determined by HPLC or at 1.0 µg/g when determined by CD-ELISA.

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