Fungal and Mycotoxin Contamination of Pearl Millet Grain in Response to Environmental Conditions in Georgia

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ABSTRACT

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Pearl millet hybrid Tift 90DAE × Tift 8677 was planted at Tifton, Georgia, on five dates in both 1990 and 1991. Grain was harvested about 33 days after anthesis. Seedborne fungi were determined from 300 seeds from each seed lot. The most frequently isolated fungi were *Fusarium semitectum* (26% of seed), *Alternaria* spp. (19%), and *Curvularia* spp. (13%). Total *Fusarium* isolations, including *F. semitectum*, averaged 46%. Isolation frequencies of several fungi were related to weather conditions 2–4 wk prior to harvest. Aflatoxins were detected from grain planted in both years, and concentrations averaged 0.3 ng/g. Deoxynivalenol, nivalenol, zearalenone, and 15-acetylscirpentriol were detected only in the 1991 samples, and levels averaged 0.3, 1.2, 0.3, and 0.4 μg/g, respectively. Isolation of *F. chlamydosporum* was positively correlated ($r \geq 0.66$) with concentrations of trichothecenes and zearalenone. Although pearl millet grown in Georgia does not appear to be prone to infection by *Aspergillus flavus* and aflatoxin contamination, a potential for problems from infection by toxigenic *Fusarium* species does exist.

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is being developed for use as a feed grain crop in the southern United States, and parental inbreds have been released by the USDA-ARS and the University of Georgia (6). Pearl millet is well adapted to sandy soils with low pH and is better adapted than field corn in the southeastern United States under nonirrigated conditions. Acceptance of the crop will depend on the economic production of high-quality grain for cattle and poultry diets (4,7,18).

The quality of feed grains can be affected by seed-infecting fungi that produce mycotoxins. Grain mold of pearl millet is commonly attributed to *Curvularia lunata* (Wakk.) Boedijn, *Fusarium semitectum* Berk. & Ravenel, and *F. moniliforme* J. Sheld. (24). Because pearl millet has not been previously grown as a grain crop in the southeastern United States, few studies have examined fungal infection of the grain in this region. Luttrell (8) identified three types of head molds on pearl millet in Georgia. One type was caused by *Oidium tenellum* (Berk. & M. A. Curtis) Linder; a second by a complex of *Helminthosporium stenospilum* (Drechs.), *Exserohilum rostratum* (Drechs.) K. J. Leonard & E. G. Suggs, and *C. lunata*; and a third by unidentified species of *Fusarium*.

Grain molds were examined in greater detail by Wells and Winstead (23), whose primary concern, however, was identification of seedborne pathogens that would reduce germination and cause seedling blights. They determined that about 44% of the grain produced in Georgia was infected by fungi, and about 7% of the grain was infected by *Fusarium* species, such as *F. moniliforme* and *F. roseum* Link:Fr.

Information concerning infection of pearl millet by *Aspergillus flavus* Link: Fr. and other potentially toxigenic fungi will be useful prior to the sale of pearl millet grain and its use as feed. The objectives of these experiments were to identify the fungal flora and quantify concentrations of mycotoxins in pearl millet grain grown in Georgia and to determine the effects of environmental conditions on contamination.

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MATERIALS AND METHODS

Pearl millet hybrid Tift 90DAE × Tift 8677 was planted 3 cm deep at a rate of approximately 45 seeds per meter in four-row plots, 4.9 m long with 0.9 m spacing between rows, on 20 April, 10 May, 30 May, 20 June, and 10 July 1990 and on 22 April, 14 May, 31 May, 17 June, and 5 July 1991. These plots were established to evaluate protectant fungicide seed treatments (27; J. P. Wilson et al, *unpublished*). Five treatments with four replications were planted in a randomized complete block on each date.

All open-pollinated panicles from each plot were harvested approximately 33 days after anthesis, oven-dried at 38 C for 4 days, and threshed. After determining yields, equal quantities by volume of seed from each plot established from fungicide-treated seed and the controls within dates of planting were bulked and mixed. We assumed that fungicide seed treatments would not affect the fungal flora of mature grain. Therefore, grain from all seed treatment and control plots within a date of planting was bulked and is hereafter referred to as a seed lot.

A total of 300 seeds from each lot were surface-sterilized for 1.5 min in a 0.05% NaOCl solution. Unrinsed seeds were plated onto V8 juice agar (20% V8 juice and 1.5% NaOH), incubated at 25 C under continuous fluorescent lighting, and examined after 4–7 days. Fungi growing from seeds were examined microscopically for conidial and sporophore morphologies and identified when possible. Fusarium species were subcultured onto potato-dextrose agar, and other unidentified fungi were subcultured onto V8 juice agar to aid in identification.

Grain from each lot was analyzed for mycotoxin contamination. Mycotoxin analyses included assays for fumonisins, aflatoxins, nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, diacetoxyscirpenol, 3-acetyldiacetoxyscirpenol, scirpentriol, 15-acetoxyscirpentriol, T-2 toxin, iso T-2 toxin, acetyl T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, neosolaniol, zearalenol, and zearalenone.

Fumonisin assays were performed by the method of Ross et al (15). For aflatoxin assays, grain samples were ground to pass through an 850-μm sieve. Aflatoxins from 50-g samples were extracted by methods described by Thean et al (20). In 1990, aflatoxins were quantified by high-performance liquid chromatography by the postcolumn iodine deriva-

tization method (2). In 1991, aflatoxins were determined by the fluorometer (Vicam, Somerville, MA) method (21).

Additional mycotoxins were assayed by a modification of the procedure of Cohen and Lapointe (3). A total of 50 grams of grain were mixed with 250 ml of a methanol/water mixture (1:1) and shaken for 30 min. The extract was clarified with ammonium sulfate and Celit 545, filtered, and extracted with ethyl acetate. After evaporating the ethyl acetate, the sample was passed through a 1-g florisil column. The final residue was dissolved in 3 ml of chloroform/methanol (7:3) for subsequent derivatization. Afterward, 1 ml was evaporated and derivatized by the method of Rizzo et al (14), in which the residue was treated with 100 μl of TMSI (*N*-trimethyl-silylimadazole) in a reaction vial for 1 hr at 65 C. Isooctane (1 ml) with internal standards was added to the reaction vial and mixed, and 1 ml of deionized water was added. The isooctane–water mixture was vortexed and centrifuged. The isooctane layer was analyzed by gas chromatography and mass spectra. Mass spectra (select ion monitoring) of the samples were obtained on an INCOS 50 system quadrapole mass spectrometer (Finnigan Corp., San Jose, CA) interfaced with a Hewlett-Packard 5840 gas chromatograph. Chromatographic separations were made on a 30 m × 0.25 mm (i.d.) fused silica capillary column with a 0.25-μm coating of OX17, using helium at a linear velocity of 35 mm/sec. Injections were on a 2 m × 0.53 mm guard column at 70 C. The column oven was programmed from 70 to 170 C at 25 C/min, then ramped from 170 to 300 C at 5 C/min and held for 4 min. Mass spectrometer conditions were ion source temperature of 175 C, transfer line at 300 C, and ionizing voltage of 70 eV EI (electron ionization). The scan descriptors were set to find three to five fragments of each trichothecene within the specified retention window. The trichothecene standards were prepared on a weight-to-volume basis.

Weather data collected at Tifton, Georgia, were summarized for 2-, 3-, and 4-wk intervals prior to the corresponding date of harvest for each grain lot. Days with rain, total rainfall, and average maximum and minimum air temperatures at 1.52 m, net evaporation, maximum and minimum relative humidities, and solar radiation were determined. Pearson's correlation coefficients (17) among environmental conditions, isolation frequencies of fungi, and mycotoxin levels were calculated. Specific periods prior to harvest that had most significant correlations with fungal isolation and mycotoxin levels were determined.

RESULTS

The most frequently isolated fungi were *F. semitectum* (isolated from 26% of the seed across all lots), *Alternaria* spp. (19%), and *Curvularia* spp. (13%) (Table 1). Within each year, fungal flora on grain harvested from different plantings did not differ greatly; however, the percentages of grain infected increased from the first to the last planting dates in 1990.

Differences in fungal populations and levels of infection were observed between the two years and were likely affected by rainfall differences. Early-season drought in 1990 may have reduced levels of infection. Rainfall in July, August, and September averaged 56 mm below normal each month (compared with a 65-yr average). Rainfall in October was 81 mm greater than average. In 1991, 50 mm more rain fell than the average for July, whereas rainfall in August, September, and October averaged 50 mm below normal.

Several correlations between means of environmental conditions and infection by the fungi were significant (Table 2). Infection by species of *Alternaria* and *Epicoccum* was favored by high net evaporation and low minimum and maximum relative humidities. Infection by *Phyllosticta penicillariae* Speg., *Gloeocercospora sorghi* Bain & Edgerton ex Deighton, miscellaneous "*Helminthosporium*" species, *E. rostratum*, and *Drechslera dematioidea* (Bubák & Wroblewski) Subramanian & P. C. Jain was favored by high rainfall frequency or total rainfall and high minimum relative humidities. Infection by *Curvularia* species, *Bipolaris setariae* (Sawada) Shoemaker, *F. semitectum*, and miscellaneous *Fusarium* species was favored by low maximum temperatures, net evaporation, and radiation. Infection by *F. moniliforme* was not correlated with any environmental measurements in these experiments.

Low concentrations of mycotoxins were detected in the samples (Table 3). Aflatoxin levels averaged 0.3 ng/g. Although *A. flavus* was not isolated from the 300 seed samples, it was detected during plating of an additional 1,000 seed samples from 1990 (28). Concentrations of deoxynivalenol, nivalenol, zearalenone, and 15-acetylscirpentriol averaged 0.3, 1.2, 0.3, and 0.4 μg/g, respectively, in the 1991 samples. These mycotoxins were not detected in grain from 1990.

Concentrations of detected trichothecenes and zearalenone were positively correlated with isolation of *F. chlamydosporum* Wollenweb. & Reinking (Table 4), suggesting that this fungus may be responsible for the contamination in 1991. Miscellaneous and total *Fusarium* isolations were also correlated with concentrations of nivalenol. Although *F. semitectum* was the most frequently isolated *Fusarium* species, it was not correlated with concentrations of mycotoxins. Average net evaporation 2 wk before grain harvest were negatively

Table 1. Fungi isolated from pearl millet grain harvested from plants established at different dates of planting at Tifton, Georgia, in 1990 and 1991

	Percent isolation[a] from date of harvest										
	1990					1991					
Fungus	24 July	19 Aug.	10 Sept.	24 Sept.	15 Oct.	24 July	11 Aug.	2 Sept.	17 Sept.	8 Oct.	Mean
Alternaria spp.	18.7	39.3	41.7	61.3	22.0	2.0	1.3	1.0	2.0	3.3	19.3
Curvularia spp.	4.0	5.7	9.0	10.7	14.3	14.0	23.4	16.6	12.7	16.0	12.6
Phyllosticta penicillariae	0.7	1.7	4.0	1.3	0.7	13.7	19.0	23.7	8.7	3.0	7.7
Exserohilum rostratum	3.0	4.3	3.3	7.3	7.0	11.0	12.7	3.7	2.3	1.0	5.6
Bipolaris setariae	0.0	0.0	0.3	1.3	2.3	2.3	5.0	4.0	2.0	5.0	2.2
Drechslera dematioidea	0.0	1.0	0.7	1.0	0.3	2.3	2.3	0.3	0.0	0.3	0.8
Misc. *"Helminthosporium"* spp.	2.0	0.7	0.7	0.0	0.3	4.3	2.0	1.7	0.7	1.0	1.3
Penicillium spp.	0.0	0.3	0.7	0.3	0.0	0.7	0.7	6.7	3.3	0.7	1.3
Epicoccum spp.	0.7	1.3	2.7	3.7	2.3	0.0	0.0	0.0	0.0	0.3	1.1
Cladosporium spp.	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.7	1.0	0.2
Gloeocercospora sorghi	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.2
Fusarium semitectum	5.7	9.3	24.3	12.7	55.7	21.7	19.7	27.0	35.7	44.7	25.7
F. chlamydosporum	1.3	3.0	1.7	2.7	4.3	5.3	13.7	23.0	23.3	7.3	8.6
F. equiseti	0.3	0.0	1.7	0.7	1.7	17.3	1.7	1.0	0.3	0.7	2.5
F. moniliforme	0.0	2.3	2.3	0.7	0.3	0.0	1.3	0.0	0.3	0.7	0.8
Misc. *Fusarium* spp.	0.3	1.0	4.7	2.7	8.0	9.0	10.6	10.7	16.0	19.4	8.2
Seed without fungi	66.7	38.3	13.0	6.7	0.0	15.7	12.0	1.3	11.3	11.3	17.6

[a] Values are percentages of isolations from 300 seed of each planting date. Isolation of more than one fungus from a single seed was common.

correlated with concentrations of several trichothecenes and zearalenone, respectively, in the grain.

DISCUSSION

The fungal flora identified in this study differ from that reported by Wells and Winstead (23), who examined infection of 18 seed lots of pearl millet produced in Georgia. Compared to our results, they found a greater percentage of seeds infected with *B. setariae* and a lower percentage infected with *Alternaria* and *Fusarium* species. Most of their *Fusarium* isolates were *F. moniliforme*, whereas in our experiments *F. semitectum* was the most frequently identified species at all planting dates.

The discrepancy between percentages of *B. setariae* isolated from grain may be due in part to the relative susceptibility of the different pearl millet cultivars to the pathogen. Wells and Burton (22) reported that leaf spot of pearl millet caused by *B. setariae* was common on cultivars evaluated at the time, but the most common foliar blight of maturing plants of hybrid Tift 90DAE × Tift 8677 is caused by *E. rostratum* (J. P. Wilson, *unpublished*). We found greater infection of grain by *E. rostratum* than by *B. setariae*. It is likely that inoculum levels of *E. rostratum* were greater in our plots than in fields from which the samples of Wells and Winstead (23) were obtained.

The relatively high isolation of *P. penicillariae* may indicate how the pathogen was introduced into the United States. *P. penicillariae* is a common foliar pathogen in Africa (16) and was recently identified in Georgia (25) and in leaf

Table 2. Pearson's correlation coefficients between environmental conditions and fungal infection of 10 lots of pearl millet grain grown at Tifton, Georgia, in 1990 and 1991

Fungus	Days with rain[a]	Total rain[b]	Air temperature		Net evaporation[e]	Relative humidity[f]		Radiation[g]
			Max.[c]	Min.[d]		Max.	Min.	
Alternaria spp.	...[h]	...	0.73*[i]	...	0.82**	−0.63+	−0.72*	...
Epicoccum spp.	...	...	...	...	0.64*	−0.80**	−0.75*	...
Fusarium chlamydosporum	...	...	...	...	−0.78*	0.56+	...	...
Penicillium spp.	...	...	...	...	−0.57+	...	...	...
F. equiseti	...	0.72*	...	...	...	...	...	...
Cladosporium spp.	...	−0.57+	−0.62*	−0.71*	...	...	...	...
Phyllosticta penicillariae	0.62+	...	...	...	−0.71*	0.66*	0.83**	...
Gloeocercospora sorghi	0.60+	...	...	...	...	...	0.61*	...
Misc. *"Helminthosporium"* spp.	0.64*	0.73*	...	...	...	0.65*	0.79**	...
Exserohilum rostratum	0.63+	0.89**	...	...	...	...	0.62+	...
Drechslera dematioidea	0.70*	0.83**	...	...	...	...	0.64*	...
Curvularia spp.	...	...	−0.69*	...	−0.87**	...	0.65*	−0.77**
Bipolaris setariae	...	...	−0.85**	...	−0.91**	...	0.60+	−0.79**
F. semitectum	...	...	−0.75*	−0.70*	−0.57+	...	...	−0.81**
Misc. *Fusarium* spp.	...	...	−0.88**	−0.65*	−0.87**	...	...	−0.82**
No infection	...	...	...	...	0.61+	...	...	0.77**

[a] Within 28 days of harvest.
[b] At 28 days before harvest.
[c] Average maximum air temperature 1.52 m aboveground 28 days before harvest.
[d] Average minimum air temperature 1.52 m aboveground 14 days before harvest.
[e] Average net evaporation 21 days before harvest.
[f] Average maximum and minimum relative humidity 28 days before harvest.
[g] Average radiation 28 days before harvest.
[h] Nonsignificant correlation.
[i] +, *, and ** = Significant correlations at $P = 0.10$, 0.05, and 0.01, respectively.

Table 3. Mycotoxins identified in pearl millet grain samples grown at Tifton, Georgia, in 1990 and 1991

Mycotoxin	Amounts[a] detected from harvested grain									
	1990					1991				
	24 July	19 Aug.	10 Sept.	24 Sept.	15 Oct.	24 July	11 Aug.	2 Sept.	17 Sept.	8 Oct.
Aflatoxins	1.8	...[b]	0.3	...	...	...	...	...	...	1.0
Deoxynivalenol	...	...	...	...	...	...	0.8	0.4	0.3	...
Nivalenol	...	...	...	...	...	...	1.2	1.7	2.2	0.8
Zearalenone	...	...	...	...	...	...	0.5	1.0	...	...
15-Acetylscirpentriol	...	...	...	...	...	...	...	0.7	1.1	...

[a] Aflatoxins are expressed in ng/g; the others, in μg/g.
[b] No mycotoxins detected at the detection thresholds.

Table 4. Pearson's correlation coefficients between mycotoxin contamination with isolation of *Fusarium* species and environmental conditions 2 wk before harvest

Mycotoxin	Fungal isolation			Environmental conditions		
	F. chlamydosporum	Misc. *Fusarium*	Total *Fusarium*	Days w/rain	Net evaporation	Minimum relative humidity
Deoxynivalenol	0.69*[a]	...[b]	...	...	−0.62+	0.57+
Nivalenol	0.97**	0.70*	0.61+	...	−0.71*	...
Zearalenone	0.66*	...	...	...	−0.55+	...
15-Acetylscirpentriol	0.88**	...	...	−0.55+	...	...

[a] +, *, and ** = Significant correlations at $P = 0.10$, 0.05, and 0.01, respectively.
[b] Nonsignificant correlation.

samples of Tift 90DAE × Tift 8677 from Texas (J. P. Wilson, *unpublished*). It has become a relatively important component of the leaf blight complex of pearl millet in Georgia (26). Isolation from seeds has not been reported previously. Spegazzini (19) found infection by *P. penicillariae* on leaves and husks (probably the floral bracts) of pearl millet. Infection of husks would put the pathogen in close proximity to seed. In India, Mathur et al (11) isolated a *Phoma* species from pearl millet grain that may be the same pathogen we have identified as *P. penicillariae*. It is likely that the pathogen may have been introduced into the United States on imported seeds.

F. semitectum was frequently isolated from pearl millet grain. *F. chlamydosporum* and *F. moniliforme* were isolated in low frequencies from nearly all seed lots. These fungi have the potential to produce mycotoxins (9). Toxigenic or potentially toxigenic fungi have been isolated from pearl millet grain in the Republic of South Africa (10), Nigeria, Zimbabwe (13), and India (5). *Aspergillus* species have been identified as primary fungal contaminants of pearl millet grain used for food by the Paharia tribe in India (12), and much of the grain was contaminated with aflatoxins. We detected low levels of aflatoxin in our samples, but environmental conditions in southern Georgia may not be conducive for aflatoxin production on pearl millet grain. *A. flavus* infection of corn and aflatoxin contamination were severe in 1990 (28; and D. M. Wilson, *unpublished*), but levels were insignificant in pearl millet.

The low frequency of infection by *A. flavus* encourages development of pearl millet as a grain crop. The low levels we detected suggest that in the southeastern United States, aflatoxin may not be as serious of a problem in pearl millet grain as it is in corn.

On the basis of isolation frequency and toxigenic potential (9), *F. chlamydosporum* and other *Fusarium* species may be potentially troublesome fungi on pearl millet grain in the southeastern United States. Levels of deoxynivalenol and zearalenone detected in our samples are sufficient to result in feed refusal and estrogenic effects in swine (9).

A trend for greater infection of grain from the later planting dates was evident in the 1990 experiment. Sufficient rainfall, and not a lack of inoculum, was apparently the limiting factor in development of grain mold of sorghum in India (1). Although rainfall was important for infection by some fungi in our study, ambient temperature, net evaporation, relative humidity, and radiation were also correlated with fungal infection. Given the data obtained thus far, planting of the crop to allow grain fill and maturation during July or early August may coincide with generally lower rainfall and greater ambient temperatures, net evaporation, and radiation. Grain production under these environmental conditions may significantly reduce problems resulting from potential infection by toxigenic *Fusarium* species.

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