Influence of Relative Humidity on Production of Aflatoxin in Rice by Aspergillus parasiticus

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ABSTRACT

At 30 C, invasion and colonization of rice inoculated with *Aspergillus parasiticus* increased with storage humidity. At relative humidities (RH) of 70, 75, and 80%, 15 to 30% of the kernels were infected.

More rapid invasion and colonization by *A. parasiticus*, greater accumulations of aflatoxins, and greater activity by species of the natural mycoflora accompanied increases in the storage RH. Decreases in aflatoxin accumulations detected during late storage appeared to be associated with increased activity by species of the *A. glaucus* group. The latter relationship indicated that more attention should be given to the effect of competitive species during storage.

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Additional key words: Aspergillus flavus group, moisture contents.

Aspergillus flavus Link. was associated with the production of aflatoxins in peanuts by Sargeant et al. (18, 19). Subsequent research has shown that aflatoxins are produced in a wide variety of agricultural products, including rice. Several surveys and reviews of the aflatoxin problem (10, 12, 14, 25) have been published. Calderwood & Schroeder (7) reported that alfatoxins developed in undried rough rice stored in aerated bins. Schroeder & Sorenson (21) reported that species of the *A. flavus-oryzae* group, now the *A. flavus* group (17), were the dominant Aspergilli found in all levels in experimental bins of stored rice in which the moisture content ranged from 14 to 18%. *A. flavus* frequently is found in seeds with a moisture content in equilibrium with a relative humidity (RH) of 85 or 90%. In rice, a starchy cereal seed, such a moisture content could be expected to range from approximately 18 to 20% (10). However, *A. flavus* is reported to grow at RH of 80% at 30 C (23). Austwick & Ayerst (4) stated that the organism could grow at or above 75% RH at a temperature range of 30 to 38 C. Their reports emphasize the extreme variability of response to conditions frequently found within a species or species group. As further evidence of variability, Armbrecht et al. (2) reported that for the same strain of *A. flavus*, mycelial growth and spore production were not always uniform on the same substrate. From 284 randomly selected isolates of the *A. flavus* group from rice in commercial channels in five states, we observed that 16 isolates did not produce aflatoxins in sterile moist rice (6). We also found that 63% of the 268 toxin-producing isolates did not produce aflatoxins G_1 or G_2 on the sterile rice.

In many studies reporting the production of aflatoxin and/or the mycoflora associated with rice, the rice had been preconditioned to a desired moisture level by adding water (6, 9). In other studies naturally infected, dried or undried rough rice was placed in aerated experimental bins (7, 21). In a previously reported study (20) and in the present experiments, dry rough rice was inoculated with dry conidia of the species being studied. To simulate natural invasion, the dry rice then was placed in selected storage atmospheres and allowed to adjust to the conditions.

Aspergillus parasiticus Speare (17) is one of the more toxicogenic species of the *A. flavus* group and produces all four major aflatoxins. Little is known of the effects of various factors upon the activity of this species in stored rice.

MATERIALS AND METHODS.—Inoculum for these experiments was grown from an isolate of *A. parasiticus* originally isolated from Texas rice. Dry conidia were produced and harvested as previously described (5).

Dry conidia were transferred with a spatula to large jars containing dry rough rice. The jars were closed and shaken to distribute the conidia and the inoculated rice was transferred to a wire screen basket (100 g of rice per basket). The baskets were suspended over water or saturated aqueous salt solutions in wide-mouth quart Mason jars. Each jar was closed with a lid containing a 14-mm (0.5-inch) diam hole tightly plugged with cotton. Measurements with a "Hygrophil" thermistor psychrometer verified that these chambers maintained the storage humidities essentially the same as reported for closed systems (8, 13, 24). The solutions and their respective humidities at 30 C were: water, 100%; $BaCl_2$, 90%; $NaCO_3 \cdot 3H_2O$, 87-88%; KCl, 85%; $(NH_4)_2SO_4$, 80%; NaCl, 75%; and $CH_3COONa \cdot 3H_2O$, 70%.

The inoculated rice and the respective noninoculated controls were stored simultaneously in separate containers in a controlled-temperature cabinet at 30 C.

Moisture contents of the rough rice were determined by the official two-stage air oven method (1).

The percentage of kernels infected by the inoculated species and by species of the natural mycoflora were determined by plating the kernels on malt-salt agar (2.0% malt extract, 1.8% agar, 7.5% NaCl/liter). The rough rice kernels were surface disinfected in 1.0% NaOCl (20% aqueous solution of commercial bleach) for 1 min followed by a 1-min rinse in sterile water. The kernels then were placed in sterile water in sterile petri dishes. Individual surface-disinfected kernels were transferred aseptically to malt-salt agar in petri dishes (four plates of 50 kernels for each sample tested). The petri dishes were incubated at room temperature for 5 to 7 days to permit identification of the fungi growing from the kernels.

After incubation, samples to be assayed for aflatoxins were autocalved for 30 min at 1.05 kg-force/cm^2 (15 psi). After drying at room temperature to remove excess moisture, the rice was ground in a Wiley Mill to pass a 20-mesh wire screen; 50 g were transferred to a 500-ml Erlenmeyer flask and extracted with 250 ml aqueous acetone (70%, v/v) following the method of Pons & Goldblatt (16). The extract residues were resolved and quantitative estimates of aflatoxins were made with thin-layer chromatography (TLC). Under long-wave ultraviolet light, four concentrations of the unknown were visually compared to an equal volume of the standard solution following development on 0.25-mm thick Silica Gel G-HR, in chloroform containing 3% methanol (v/v).

RESULTS.—The moisture contents of the rough rice (initial moisture content ca. 12.9%) increased rapidly during the first 7 days (Fig. 1). At RH of 90% or less, equilibrium moisture contents were approached in 7 to 21 days. At 100% RH, moisture content of rice continued to increase through 42 days to 23.0%. After 42 days at 90% RH, moisture content was 17.2%.

Species of the *A. flavus* group were not observed in the noninoculated controls. Species of the *A. glaucus* group (17) were recovered from more than 90% of the plated surface-disinfected kernels after 21 days at RH of 87% or higher at 30 C. Eighty percent of the kernels were infected after 21 days at 85% RH

and 28 days at 80% RH. After 28 days, 70% of the kernels were infected at either 70 or 75% RH. No aflatoxins were detected in the noninoculated rice.

A. parasiticus infected kernels of inoculated rice stored at RH of 75% or higher (Table 1). After 7 and 14 days, the percentage increased as the RH increased. Colonization of the kernels by the inoculated species was slow in rice stored at 75 or 80% RH. Frequently only one or two conidiophores of *A. parasiticus* were found on infected kernels. In contrast, after 14 days in RH of 85% or higher, infected kernels were rapidly colonized as indicated by the high prevalence of conidiophores and the large affected area of the kernels.

Species of the *A. glaucus* group, the only highly prevalent naturally occurring species in the inoculated rice, infected increasingly greater percentages of kernels as the storage RH was increased and colonization developed more rapidly at the higher RH. The *A. glaucus* group became the more prevalent fungi after 28 days in 100% RH, 21 days at 90%, and after 14 days at 85% or less even though colonization was slow at lower RH.

Aflatoxins were detected in inoculated rice stored at all of the RH in these experiments. Only traces of aflatoxin B_1 were detected after 7 and 14 days in rice stored at 75 or 80% RH. Traces of aflatoxin G_1 also were detected on these sampling dates in rice stored at 80% RH. The quantities of toxins detected in rice stored at 85% or higher RH increased as the atmospheric humidity increased (Table 2).

These experiments were repeated with RH ranging from 70 to 90%. Again, the *A. glaucus* group were the dominant fungi in the noninoculated rice and the percentage of infected kernels followed the trend

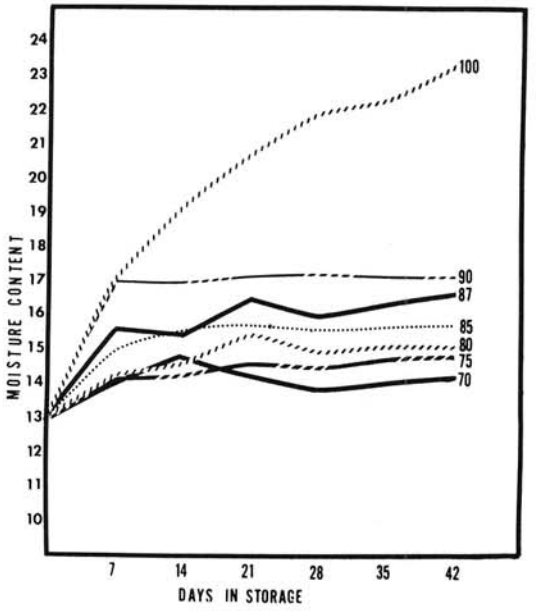

Fig. 1. Moisture content of 'Belle Patna' rough rice stored at 30 C in several relative humidities.

TABLE 1. Precent infection of rice inoculated with *Aspergillus parasiticus* spores and stored at 30 C and a range of relative humidities

Relative humidity (%)	Infection of rice grains (%) after storage			
	7 days	14 days	21 days	28 days
100	93.0	95.5	97.0	91.5
90	81.0	87.5	83.0	79.0
85	73.0	71.5	66.5	68.5
80	64.0	68.5	70.5	75.0
75	61.5	66.5	66.0	69.0

observed in the preceding experiments. Species of the *A. flavus* group and aflatoxins were not detected in noninoculated rice.

The percentages of kernels of inoculated rice infected by *A. parasiticus* were less than those observed in the preceding experiment (Fig. 2). Larger percentages of kernels of inoculated rice appeared to be infected by species of the *A. glaucus* group during the early stages of storage and colonization by these species appeared to be more rapid than observed in the preceding experiment.

Although quantities were smaller than in the preceding experiments, aflatoxins were found in rice stored at each RH. Traces of aflatoxin B_1 were detected after 42 days in rice stored at either 70 or 75% RH. Measurable quantities of aflatoxin B_1 were found after 28 days in rice stored at 80, 85, or 87-88% RH. All four aflatoxins were found only in the inoculated rice stored at 90% RH. Aflatoxin B_1 were detected after 21 days and all four toxins after 28 days. The largest quantities were detected after 35 days (B_1 = 63 μg/kg, B_2 = 11 μg/kg, G_1 = 571 μg/kg, and G_2 = 46 μg/kg).

DISCUSSION.—Experience in our laboratory showed the surface-disinfection method to be effective. No kernels of noninoculated rough rice previously shown to be free of *A. flavus* were infected when plated on malt-salt agar after experimental storage.

Moisture contents of the rice in these experiments did not always agree with those reported by other authors (9, 10, 11, 13). The differences observed were believed to reflect hygroscopic differences attributable to variety and response of mycoflora activity.

According to Coleman & Fellows (11), the hygroscopic moisture contents in corn, oats, barley, rice, and rye do not differ greatly from those of wheat. Pixton & Warburton (15) reported that approximately 30 days were required to obtain complete equilibrium in wheat and, when grain was gaining moisture, more than 90% of the water absorbed was taken up within 5 to 14 days. In our experiments, more than 90% of the water absorbed by rice stored at RH of 90% or less was absorbed during the first 7 days. In rice stored at 100% RH, the moisture content of the rice increased through 28 and 42 days; concurrently, fungi continued to grow on or in the kernels. The effect of the fungi on the moisture

TABLE 2. Aflatoxins detected in rough rice, inoculated with *Aspergillus parasiticus*, after storage at 30 C

Relative humidity (%)	Aflatoxin	Aflatoxin levels (μg/kg) after storage 7 days	14 days	21 days	28 days
100	B_1	357	8,571	1,143	714
	B_2	- [a]	1,143	229	143
	G_1	214	28,572	5,714	3,571
	G_2	86	2,286	457	571
Total		657	40,572	7,543	4,999
90	B_1	357	-	143	14
	B_2	71	-	29	-
	G_1	1,429	-	429	-
	G_2	29	-	34	-
Total		1,886	0	635	14
85	B_1	36	-	19	29
	B_2	-	-	-	-
	G_1	71	-	57	-
	G_2	-	-	14	-
Total		107	0	90	29

[a] Spaces with a dash indicate that no aflatoxins were detected or that interfering compounds precluded identification.

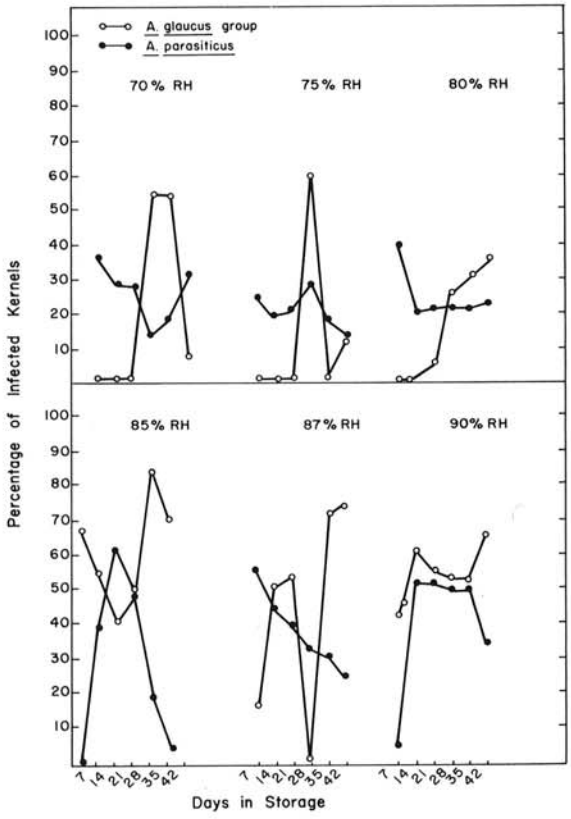

Fig. 2. Invasion of inoculated rough rice by *Aspergillus parasiticus* and by naturally occurring species of the *A. glaucus* group during storage in several relative humidities at 30 C.

content of the rice sample is not definitely known, but fungal activity might be a substantial contributor to the continued increase in moisture content of the rice in these experiments.

Increased fungal growth and activity in commodities stored at several RH led Snow et al. (22) to conclude that mold growth depended more upon RH than on moisture content of the commodity. Christensen (9) stated that the moisture contents of rice (17 to 18%) in equilibrium with a RH of 90 to 95% were ample for the growth of *A. flavus*. Armolick & Dickson (3) reported that the minimum moisture content of an agar film required for germination of conidia of *A. flavus* was 15.1% (at equilibrium with an atmosphere of 80.2% RH). They believed that grain was unlikely to mold at the minimum humidity required for germination. Abnormalities in spore germination and in germ tube development suggested that the fungi may have difficulty in establishing in tissues at the minimum limits; however, they stated that *A. flavus* produced conidiophores and conidia at moistures slightly above the minimum RH required for germination. In the present experiments, 15 to 30% of the kernels of rice stored at RH ranging from 70 to 80% were invaded by *A. parasiticus*. Invasion and growth occurred when the moisture content of the rice exceeded 14.2% moisture at 70% RH. This invasion and growth agreed with Schroeder & Sorenson (21) finding that species of the *A. flavus* group were the dominant fungi at all levels in bins in which the moisture content of rice ranged from 14 to 18%.

The sparse development of conidiophores and conidia on kernels stored at the lower RH, suggested that the fungus did have difficulty becoming established at the lower humidity levels. This observation agreed with the conclusion of Armolick & Dickson (3). However, the findings of Schroeder & Sorenson (21), and the observation of individual kernels supporting luxuriant growth of the fungus at the lower RH of these experiments, suggested that more attention should be given to the microenvironment surrounding the point of contact of the fungus and the kernel tissue. The rapid rate of infection of rice by *A. parasiticus* during the phase of rapid water uptake may reflect a microenvironment, at the site of spore germination or tissue invasion, that may differ considerably from the overall storage atmosphere. The luxuriant growth of the fungus on a few kernels also may reflect variations in the internal environment of specific kernels and the storage atmosphere.

The moisture contents determined in these experiments, as well as in those reported for grain in prior studies, represent the average of moisture contents of all the kernels or seed in a specific sample. In a random sample, some divergence from the average is expected. In kernels with a higher moisture content, the microenvironment at a given site could favor the growth of fungi. Subsequent growth of fungi could lead to increased moisture. Migration of this moisture to surrounding tissue could provide the microenvironment required to support

the rapid growth of the fungus and the apparent luxurious colonization of a relatively small number of kernels stored at lower RH. The increased activity of *A. parasiticus* in these scattered individual kernels could be reflected in finding small quantities of aflatoxins in rice stored at the lower RH in these experiments.

Increased activity of species of the natural mycoflora was observed as time in storage increased at all RH. This increased activity was particularly noticeable among species of the *A. glaucus* group in rice stored at the higher RH. Species of the *A. glaucus* group appeared to replace the inoculated species at RH of 85% or higher. Apparent decreases in the quantities of aflatoxins detected during the last 7 to 14 days of storage at 90 or 100% RH, would tend to support this observation. Schroeder et al. (20), Calderwood & Schroeder (7), and Christensen (9) have indicated that the effect of other species on the species being studied should be considered when evaluating the activity of a single species during storage.

Rapid invasion and colonization of rice by *A. parasiticus*, accumulation of aflatoxins, and the increased activity of species of the natural mycoflora as the RH of the storage atmosphere increased emphasize the necessity of maintaining rice, and other grains, at low moisture contents.

The conclusions based on this research were that the invasion of rough rice by *A. parasiticus* and the subsequent production of aflatoxins were controlled by the RH. Conditions within individual kernels of rough rice may provide a microenvironment favorable to the growth of *A. parasiticus* and the production of aflatoxins in rice stored at RH lower than that believed to be the limiting humidity. Although humidity influences the activity of *A. parasiticus* in rough rice, the influence of other factors such as competition from other species must be considered.

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