Influence of Aspergillus chevalieri on Production of Aflatoxin in Rice by Aspergillus parasiticus

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ABSTRACT

Small quantities of aflatoxin B_1 were detected in rice inoculated with *Aspergillus parasiticus* after 14 through 21 days at 85% relative humidity and 30 to 35 C. Larger quantities of aflatoxin B_1 and in addition B_2, G_1, and G_2 were detected after storage at 25 C for 28 days or longer. No aflatoxins were detected at 85% relative humidity, when the rice was simultaneously inoculated with spores of both *A. parasiticus* and *A. chevalieri*. Invasion and production of aflatoxin was rapid in rice inoculated with only *A. parasiticus* in each of the three temperatures at 100% relative humidity. *A. parasiticus* invaded rapidly but considerably smaller quantities of aflatoxins were produced when rice was simultaneously inoculated with both species at 100% relative humidity. Reduction in aflatoxin B_1 ranged from 99% at 25 C, 100% at 30 C, to 95% at 35 C.

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Additional key words: fungal competition, mycotoxin metabolization.

Schroeder & Hein (9) reported that optimal temperatures for in vitro aflatoxin development by *Aspergillus flavus* Link.appeared to be associated with accelerated growth of the fungus. They suggested that accumulation of aflatoxins was affected by the increased ability of the fungus to metabolize the toxins. Initially this hypothesis appeared to be substantiated by our experiments with stored rice. As our studies progressed and the prevalence of other species of the natural mycoflora became evident, the data suggested that their activities might modify the invasion of rice kernels and the production of aflatoxins by *Aspergillus parasiticus* Speare. In jointly infected kernels, the activities of species of the natural mycoflora frequently resulted in replacement of the inoculated species by one or more naturally occurring species. Further, toxin accumulations were not always directly related to the incidence of kernels infected by *A. parasiticus*. Similar observations were made in separate and independent studies by Calderwood & Schroeder (3) and Schroeder et al. (8).

Christensen (5) reported that in one sample of 'Nato' rice stored in conditions favorable for the growth of *A. flavus-oryzae, Penicillium* spp. scarcely increased. In the second sample, however, Penicillia became the dominant fungi. He concluded that in the presence of a varied mycoflora on or within the kernels of rough rice, under conditions allowing several species to develop, the predominance of any one species depends upon the interactions of unknown ecological factors grouped under the term "competition". Study of the competitive activity of several fungi is an important area that has been largely neglected because of the many possible combinations of microorganisms and the difficulty in accurately measuring the activity of a particular species.

In previous experiments, massive inoculations with spores of *A. parasiticus* apparently permitted measurement of the activity of *A. parasiticus* by quantifying the aflatoxin accumulating in rice stored under various atmospheric conditions. Consequently, a simultaneous massive inoculation with spores of two species appeared to offer a feasible method of studying the effect of one species upon a measurable attribute of the other.

In earlier experiments, *A. chevalieri* (Mangin) Thom & Church (7) was the most prevalent species of the *A. glaucus* group isolated from the natural mycoflora of rough rice. This paper reports experiments to evaluate the effect of competition of simultaneously introduced *A. chevalieri* on the production and/or accumulation of aflatoxin by *A. parasiticus* in experimentally stored rice.

MATERIALS AND METHODS.–Conidia of *A. chevalieri* and *A. parasiticus* were grown and harvested by the method of Boller (2). After harvest, the conidia were stored in separate sterile jars. Approximately equal volumes of the dry spores of each species were transferred to a sterile glass vial with sterile pipets. The vial was closed with a sterile rubber stopper and shaken to mix the spores. The spores were then sprinkled over the surface of 'Belle Patna' long-grained rough rice (moisture content 11.2%) in a Patterson-Kelley twin-shelled blender and mixed for 20 min to distribute the spores throughout the rice. Aliquants of 100 g of the inoculated rice was transferred to wire screen baskets suspended in wide-mouth quart jars containing either water or a saturated aqueous solution of KCl to maintain relative humidities of 100 and 85%, respectively (4). All jars were closed with cotton-plugged perforated lids. Inoculated rice and the respective noninoculated controls were stored simultaneously in controlled temperature cabinets at 25, 30, and 35 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% salt/liter). The rough rice was surface-sterilized in 1.0% Na-hypochlorite (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-sterile 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 autoclaved for 30 min at 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 (6). The extracts were resolved and the aflatoxins quantified by thin-layer chromatography (TLC). Under longwave ultraviolet light, four concentrations of the unknown were visually compared to an equal volume of the standard solution following development on Silica Gel G-HR, 0.25 - mm thick in chloroform containing 3% methanol (v/v).

RESULTS.– *Studies at 85% relative humidity.*–In the noninoculated rice stored at 85% relative humidity, species of the *A. flavus* group were not observed and no aflatoxins were detected in rice stored at 25, 30, or 35 C. At all temperatures, species of the *A. glaucus* group were the dominant fungi, infecting more than 70% of the kernels after 42 days. After 42 days, species of the *A. candidus* group (7) infected 33% of the kernels at 25 C, 35% at 30 C, and 53% at 35 C. However, colonization of the kernels by *A. candidus* was slow compared to the colonization by species of the *A. glaucus* group.

In rice inoculated only with *A. parasiticus*, this species invaded more than 80% of the kernels after 14 days at each temperature. After 42 days at 25 C, 90% of the kernels were still infected, but the percentage of kernels infected at 30 or 35 C had decreased to approximately 60%. Concurrent infection by species

of the *A. glaucus* group increased with time in storage. At 30 or 35 C, 80% of the kernels were infected by members of this group when rice was stored longer than 28 days. At 25 C, the percentage of kernels infected by these species of the natural mycoflora decreased from 75% after 35 days to approximately 50% after 42 days.

Small quantities of aflatoxin B_1 (trace to 10 μg/kg) were detected in rice inoculated with *A. parasiticus* after 14 and 21 days at 30 or 35 C and none was detected in rice at these temperatures after longer storage periods. At 25 C, a trace of aflatoxin B_1 was detected after 14 and 21 days, 50 μg/kg after 28 days, but less than 20 μg/kg after 42 days. All four toxins were detected in rice stored 28 days or longer. After 42 days, the ratio of aflatoxin G_1 to B_1 was approximately 2.5 times that after 28 days.

In rice simultaneously inoculated with *A. parasiticus* and *A. chevalieri*, none of the kernels stored at 25 or 30 C and only 5% of the kernels at 35 C were infected by *A. parasiticus* after 42 days at 85% relative humidity. The incidence of this species was greatest after 7 days when 10% of the kernels at all temperatures were infected. Concurrently, *A. chevalieri* infected 60% or more of the kernels after 14 days and more than 90% of the kernels after 42 days. Although *A. candidus* was observed frequently on noninoculated rice, less than 1% of the kernels stored at 85% relative humidity were infected in the rice simultaneously inoculated with *A. parasiticus* and *A. chevalieri*. No aflatoxins were detected in the rice simultaneously inoculated with both species.

Studies at 100% relative humidity.—Species of the *A. flavus* group were more prevalent in noninoculated rice at 100% than at 85% relative humidity. The percentage of kernels infected by these species ranged from 10% at 25 C to 16% at 35 C after 7 days, but decreased to less than 5% at each temperature when rice was stored longer than 21 days. Infection by species of the *A. glaucus* and *A. candidus* groups increased with time in storage. At 25 and 30 C, species of the *A. glaucus* group were the dominant fungi throughout storage. However, *A. candidus* was more active at 35 C and became dominant after 42 days. Traces of aflatoxin B_1 were detected after 7 days in rice stored at each of the temperatures and after 14 days in the rice stored at 35 C. No aflatoxins were subsequently detected in the noninoculated rice.

All kernels of rice inoculated only with *A. parasiticus* were infected by this species after 14 days at 25, 30, and 35 C (Table 1). At each temperature, the incidence of infected kernels remained above 90% through 42 days. At each temperature, concurrent infection by species of the *A. glaucus* group was greatest after 14 days and then decreased throughout the remaining time in storage. Colonization of the kernels showed that *A. parasiticus* was dominant throughout storage at all temperatures.

The prevalence of kernels infected by *A. parasiticus* approached 100% after 14 days at each of the three temperatures in rice simultaneously inoculated with the two species. Although more than 90% of the kernels remained infected by *A. parasiticus* through 42 days at 25 and 30 C, only 84% of the kernels were infected at 35 C (Table 1). Concurrent infection by the second species was not as rapid as that of *A. parasiticus*. However, relatively large percentages of the kernels were infected by *A. chevalieri* after 14 days at 25 and 30 C. The incidence of infection by this species was lower at 35 C than at either 25 or 30 C. At each temperature, colonization of the kernels by *A. chevalieri* decreased as time in storage increased. This species did not become dominant on kernels jointly infected by the two species. Species of *Penicillium* and *A. candidus* were present but, regardless of temperature, infected less than 10% of the kernels throughout storage.

The quantities of aflatoxins detected in rice simultaneuosly inoculated with both species were considerably less than that in rice inoculated only with *A. parasiticus* (Fig. 1). The reduction in the accumulation of aflatoxin B_1 ranged from 85% after 35 days to 99% after 42 days in rice stored at 25 C, from 90% after 14 days to 100% after 42 days at 30 C, and from approximately 30% after 7 and 14 days to 95% after 35 and 42 days at 35 C. Similar reductions were observed in the quantities of aflatoxins B_2, G_1, and G_2 when the rice was

TABLE 1. Kernels infected with Aspergilli in rice stored at 100% relative humidity as affected by composition of inoculum

Storage temperature	Storage period	Inoculated with *A. parasiticus* only		Simultaneously inoculated with *A. parasiticus* and *A. chevalieri*	
		A. parasiticus	*A. glaucus* group	*A. parasiticus*	*A. chevalieri*
		Infestation		Infestation	
	(days)	(%)	(%)	(%)	(%)
25 C	14	100.0	45.0	99.0	74.0
	28	99.5	37.0	97.5	76.0
	42	96.5	19.5	92.0	67.0
30 C	14	100.0	44.0	98.0	83.5
	28	100.0	28.0	97.0	69.0
	42	91.0	26.0	94.0	70.0
35 C	14	100.0	19.0	100.0	47.0
	28	100.0	1.0	93.0	65.0
	42	95.0	7.0	84.0	38.5

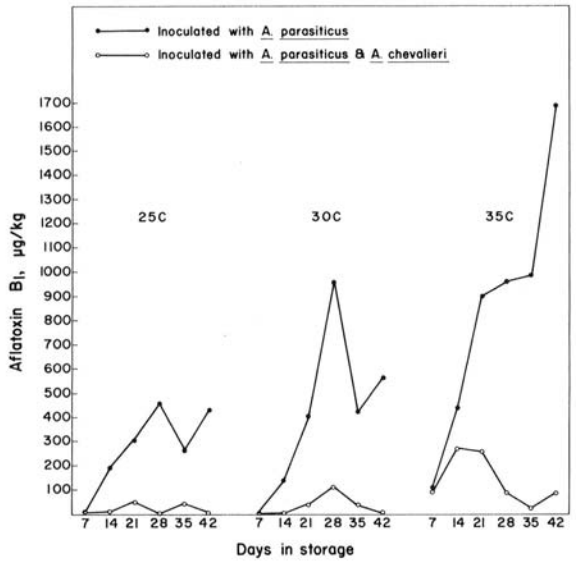

Fig. 1. The effect of simultaneous inoculation with *Aspergillus parasiticus* and *Aspergillus chevalieri* on the production and accumulation of aflatoxin B_1 in rice stored at 100% relative humidity.

inoculated with both species. The results were confirmed in duplicate experiments.

DISCUSSION.—Significant quantities of aflatoxins were detected only in rice inoculated with *A. parasiticus* and only when storage conditions were favorable for the rapid growth of the fungus. At the limiting relative humidity (85%), only small quantities of toxins were detected.

A significant change in relative quantities of G_1:B_1 observed at 25 C suggested that a metabolic conversion of B_1 to G_1 is a function of time.

Simultaneous inoculation of rice with *A. parasiticus* and *A. chevalieri*, which did not materially reduce the percent of kernels invaded by *A. parasiticus*, did reduce markedly the amount of aflatoxins produced. There are several possible explanations of this phenomenon. *A. chevalieri* may partially replace *A. parasiticus* on the jointly infected kernels, or may metabolize some aflatoxins while coexisting at the same or at neighboring infection sites. Another possibility is that *A. chevalieri* more actively competes for a nutrient essential to the production of aflatoxin. The mechanism by which the toxin accumulations were reduced is not clear. Refinement of technique and the use of biochemical procedures might provide a more accurate evaluation of the competitive activity of the several species.

The data show that infection of rice or other agricultural products by a toxin-producing strain of *A. parasiticus* is not sufficient evidence to assume the product to be contaminated with aflatoxin. They establish that competition with other fungal species is a factor affecting concentrations of aflatoxins in the products. The presence of the causal fungus even in large numbers, therefore, is not sufficient evidence to justify condemnation of the product.

These experiments support the hypothesis that simultaneous and massive inoculation of spores of two species provides a feasible method for evaluating the influence of one species upon a measurable attribute of the second species.

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