Distribution of Aflatoxin, Citrinin, and Invading Fungal Mycelium in Rice Kernels Inoculated with *Aspergillus flavus* and *Penicillium citrinum*

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ABSTRACT

The distribution of fungal mycelium and the mycotoxins aflatoxin and citrinin in individual brown rice kernels were studied. Brown and milled rice kernels were inoculated with *Aspergillus flavus* and *Penicillium citrinum* and stored at about 85 and 90% relative humidity (rh) at 28°C. Significant amounts of aflatoxins were produced by *A. flavus* in the rice stored at 85% rh for 60 days; no citrinin was detected from *P. citrinum* after 60–90 days under the same conditions. In contrast, both fungi synthesized the mycotoxins in large quantities at 90% rh for 16–60 days. Both mycotoxins were near the invading mycelia of their fungi, which were present primarily in the germ, aleurone layer, and starchy endosperm in the highly fungus-damaged rice. The mycotoxins originally present in moldy brown rice decreased during milling but were not completely removed from highly damaged kernels.

The control of mycotoxins in food and feed is important for food hygiene and animal production. We previously showed that sterigmatocystin, a carcinogenic mycotoxin, and fungal mycelia were present in the adjacent germ, aleurone layer, and starchy endosperm, and conspicuously present around the germ in individual brown rice kernels naturally infected by *Aspergillus versicolor*. Most of the mycotoxin was removed in a milling process (Takahashi et al 1984). Rice is often infested by other mycotoxin-producing fungi and contaminated with their toxins. Kurata et al (1968) showed that the *Aspergillus flavus-oryzae* group, which includes the species of aflatoxin-producing fungi, and *Penicillium citrinum*, which synthesizes citrinin, a yellowed rice toxin, are dominant fungi along with *A. candidus* in imported rice in Japan. In the major rice-producing areas of the United States, about one fourth of the isolates of *A. flavus* are capable of producing relatively large amounts of aflatoxin (Boller and Schroeder 1968). Small quantities of aflatoxin B1 and B2 (8 and 2 ppb) were found in Egyptian polished rice infected by *A. flavus* (Norizuki et al 1987). Karki et al (1979) also obtained aflatoxin B1 (15 ppb) from 12 rice samples of Nepal. Fungal invasion and mycotoxin production in cereal grains and peanut seeds are influenced by their moisture content (mc), relative humidity (rh), and other environmental factors such as temperature (López and Christensen 1967, Trenk and Hartman 1970, Diener et al 1967). Few papers report distributions of fungal mycelia and associated mycotoxins in individual rice kernels stored at different humidity levels.

This study examined the locations of the invading mycelia and mycotoxins, aflatoxin or citrinin, in individual brown and milled rice kernels when they were artificially inoculated with toxigenic *A. flavus* or *P. citrinum* and stored at about 85 or 90% rh.

MATERIALS AND METHODS

Preparation of Fungus-Infected Brown and Milled Rice

A. flavus R-1, an isolate from peanut seed, and *P. citrinum* NRRL 1843 were used as test strains capable of producing aflatoxins B1, B2, G1, and G2, or citrinin, respectively. They were cultured on moist autoclaved brown rice at 28°C for two weeks to produce the inoculum. Brown and milled rice (milling yield, 90–91%) substrates were placed in open petri dishes and gas-sterilized with propylene oxide. They were equilibrated under a controlled atmosphere of about 85 or 90% rh maintained with saturated potassium chloride solution and monobasic phosphate solution, respectively, at 28°C for two weeks. Dry spores of each fungus on the moldy rice were used as inocula by mixing them with the sterile kernels thoroughly. After removing the moldy kernels, the inoculated brown rice was stored at 85 or 90% rh. The inoculated milled rice was stored only at 85% rh. Twenty-five grams of *A. flavus*-infected rice was withdrawn 15, 30, 45, and 60 days after inoculation and storage at 85% rh, or after 5, 10, and 15 days of storage at 90% rh. Similarly, 25 g of *P. citrinum*-infected rice was taken out after 30, 45, 60, and 90 days of storage at 85% rh and after 15, 30, 45, and 60 days at 90% rh.

Moisture Content

The moisture content of each moldy rice sample was determined

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(using the method of Nagahara and Tsutsumi 1962) by heating whole kernels for 17 hr at 135°C.

Fluorescence Microscopy

The fungus-infected rice kernels were sliced longitudinally or transversely, and the location of the fluorescence due to the mycotoxin (aflatoxin or citrinin) in the sliced kernels was observed with a New Olympus Vanox photomicroscope equipped with a reflected light attachment as previously reported (Takahashi et al 1984).

Scanning Electron Microscopy

Moldy whole and sliced kernels were double-fixed with glutaraldehyde and osmium tetraoxide. They were dehydrated, critical-point dried, and coated with gold in a manner similar to that reported previously (Takahashi et al 1987). These specimens were observed with a Hitachi S-450 scanning electron microscope.

Milling, Extraction, and Determination of Aflatoxin and Citrinin

The moldy brown rice was milled for 6 min with a Kette Parest test mill. The contents of the mycotoxin, aflatoxin or citrinin, in the milled rice and the rice bran plus polish fractions were analyzed. Aflatoxin B1 and G1 were determined using the method of Kamimura et al (1985), which includes extraction with chloroform-water, purification by Florisil column chromatography, and quantitation by densitometry on a high-performance thin-layer chromatography plate. Citrinin was measured according to the method of Nakazato et al (1981). The toxin was extracted with ethyl acetate and transferred to sodium bicarbonate solution and then reextracted with chloroform. The extract so obtained was reacted with aluminum chloride to give the corresponding salt. The intensity of fluorescence due to the aluminum salt was determined by fluorospectrometry.

RESULTS AND DISCUSSION

Moisture Content

Table I shows that the moisture content of *A. flavus*-infected brown and milled rice increased gradually during storage at 85% rh. In contrast, no appreciable changes were observed in the moisture content of the *P. citrinum*-infected brown and milled rice stored under the same conditions. Under storage at 90% rh, the moisture content of both infected rices increased to about 22–23%. Trenk and Hartman (1970) described *A. flavus* growing in corn kernels and forming aflatoxin at moisture levels above 17.5% at temperatures of 24°C or higher.

Fluorescence Microscopy

Storage at 85% rh. Fluorescence microscopic observation of the longitudinal section of *A. flavus*-infected rice kernels showed that the germ portion became bright 30 days after inoculation (Fig. 1), but only dim or slight fluorescence was detected in the germ of *P. citrinum*-infected brown rice kernels. The glow of fluorescence was also found over the entire surface of *A. flavus*-infected milled rice kernels after 45 days but scarcely detected on the *P. citrinum*-infected milled rice at the end of the storage period (90 days).

Storage at 90% rh. The germ, aleurone layer, and endosperm adjacent to these tissues in the *A. flavus*-infected brown rice kernels gave bright fluorescence, just as *A. versicolor*-infected brown rice did, as reported in our previous paper (Takahashi et al 1984). The *P. citrinum*-infected rice also emitted fluorescence in the aleurone and adjacent starchy endosperm near the germ (Fig. 2) in 15 days.

The fluorescence remained conspicuous around the endosperm proximate to the germ in the milled rice kernels derived from brown rice stored at 90% rh, but its glow diminished considerably. After storage at the lower relative humidity, the derived milled rice kernels rarely or never showed fluorescence.

The color of the fluorescence detected in the *A. flavus*-infected rice was bright blue under ultraviolet light. Aflatoxin B1 and B2 emit blue fluorescence, which can be distinguished in color from fluorescent material presumably converted from kojic acid, which is bright greenish yellow in corn kernels (Fennell et al 1973). Fluorescence in the *P. citrinum*-infected rice was greenish yellow under ultraviolet light, which corresponds to that of citrinin. Brown and milled rice kernels stored under both humidity levels were coarsely ground and extracted with chloroform. The extracts were spotted on a precoated Kiesel gel plate (Merck, type 60) and were developed using the solvent system chloroform/acetone (9:1, v/v) for aflatoxins and acetone/1% phenol aqueous solution/ethyl acetate (16:3:16, v/v) for citrinin. The major fluorescent substances were identified as aflatoxins and citrinin in the *A. flavus*-infected and *P. citrinum*-infected brown and milled rice, respectively. Therefore, the fluorescent glow observed by fluorescence microscopy in the fungi-infected brown and milled rice kernels was due to aflatoxins or citrinin.

TABLE I
Moisture Content of Brown and Milled Rice Inoculated with *Aspergillus flavus* and *Penicillium citrinum* Stored at Two Humidity Levels at 28°C

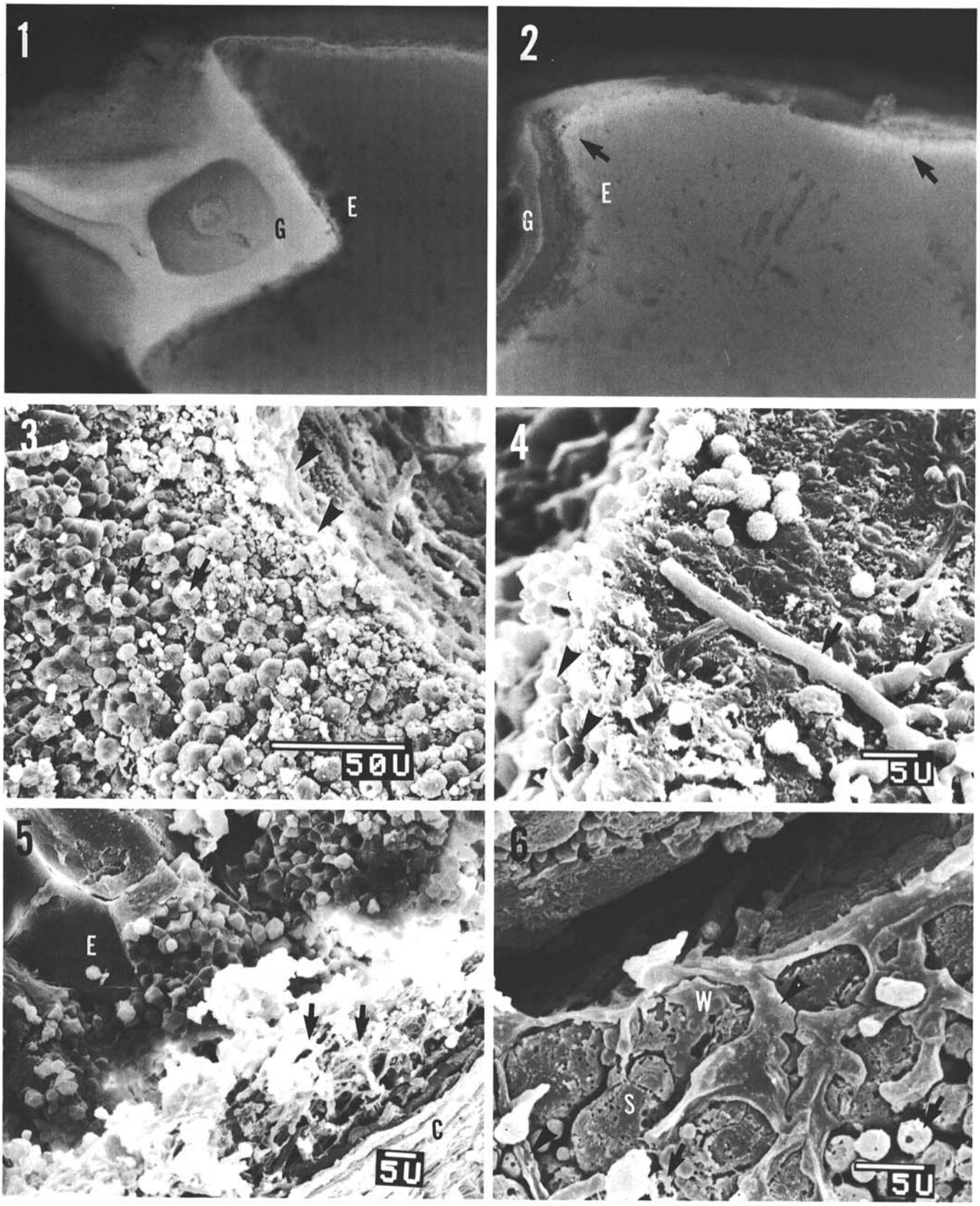
Rice Sample	% Moisture After Incubation at (no. of days)							
	0	5	10	15	30	45	60	90
Stored at 85% rh								
A. flavus infected								
brown rice	16.1[a]	···	···	16.3	16.2	16.2	17.8	···
milled rice	16.6	···	···	16.3	16.8	17.0	17.5	···
P. citrinum infected								
brown rice	16.5	···	···	16.9	16.4	16.5	16.6	···
milled rice	16.5	···	···	···	16.5	16.3	16.4	16.2
Stored at 90% rh								
A. flavus infected								
brown rice	17.6	20.0	21.2	22.3	···	···	···	···
P. citrinum infected								
brown rice	17.4	···	···	···	22.2	23.3	23.2	···

[a]Wet basis.

Scanning Electron Microscopy

Storage at 85% rh. Both fungi grew and penetrated the caryopsis coat covering the germ in 15–30 days after inoculation. Abundant growth of fungi was observed in a remnant of the vascular system on the germ coat, which agrees with findings in our previous report (Takahashi et al 1987). *A. flavus* grew well in the germ in 30–45 days, but fewer mycelia were found in the starchy endosperm (Fig. 3). *P. citrinum* developed in a similar manner in the germ, but not so actively as *A. flavus* did. No invading mycelia of the fungus were observed in the endosperm over 60 days of storage. These results were supported by those obtained from the fungi-inoculated milled rice stored under the same conditions. The hyphae of *A. flavus* extended on the milled rice kernel but they were not appreciable in the kernels (Fig. 4), which resembled those of *P. citrinum*. Previously we reported that *Eurotium rubrum* and *A. restrictus* failed to invade the starchy endosperm of brown or milled rice kernels kept at 85% rh and 28°C for 120 days (Takahashi et al 1987).

Storage at 90% rh. In contrast, *A. flavus* grew better and more easily penetrated the brown rice kernels stored at 90% rh than those stored at 85% rh. The fungal mycelia reached the endosperm through the germ and aleurone layer as quickly as three days after inoculation. *P. citrinum* also invaded the germ and aleurone but in 15 to 30 days or more. In both cases, the aleurone layer was mostly destroyed, and the aleurone grains as well as lipid bodies disappeared after fungal invasion. Mycelial masses were also detected between the caryopsis coat and the endosperm; these were conspicuous after 30–45 days in the *P. citrinum*-infected rice kernels (Fig. 5). *P. citrinum* did not develop and erode the kernels as vigorously as *A. flavus* did. The hyphae of *A. flavus* invaded first along the outer surface of the endosperm cells, causing the cells to separate, then digested their walls as well as protein bodies (Fig. 6). It appeared that *A. flavus* produced many pectolytic enzymes to degrade the cementing materials that consist mainly of pectic substances among the cells. Thus, some part of the mycelia of *A. flavus* penetrated even the inner part



Figs. 1–2. Fluorescence micrographs of sliced surfaces of *Aspergillus flavus*- and *Penicillium citrinum*-infected brown rice kernels. **1,** Section sliced longitudinally across the germ showing fluorescence due to aflatoxins in the germ (**G**) that is rare in the starchy endosperm (**E**) of an *A. flavus*-infected kernel stored at about 85% rh and 28°C for 45 days. **2,** A similar section showing a fluorescent glow due to citrinin (**arrow**) outlining the endosperm (**E**) next to the germ (**G**) of a kernel of *P. citrinum*-infected rice stored at about 90% rh and 28°C for 45 days. **Figs. 3–6.** Scanning electron micrographs of the *A. flavus*- and *P. citrinum*-infected rice kernels stored at 28°C. **3,** Surface of a section sliced longitudinally across the germ showing the fungal hyphae (**arrow head**) on the starchy endosperm (**E**) proximate to the germ of the *A. flavus*-infected kernels stored at 85% rh for 45 days. The hyphae were not found in the endosperm, and starch granules were not attacked by the fungus (**arrow**). **4,** Growing hyphae (**arrow**) surface of *A. flavus*-infected milled rice kernels after 60 days of storage. Hyphae never penetrated the kernel, and the starch granules were intact (**arrow head**). **5,** Cross section showing abundant mycelia (**arrow**) between the caryopsis coat (**C**) and the endosperm (**E**) but none in the major portion of the endosperm of *P. citrinum*-infected rice stored at 90% rh for 45 days. **6,** Longitudinal section of the endosperm showing the invading mycelia (**arrow head**) of *A. flavus* along the starchy cells of the kernel stored at 90% rh for six days. The cells were likely to separate from each other and their walls (**W**) and starch granules (**G**) as well as protein bodies (**arrow**) were partially digested by the fungus.

of the endosperm, whereas most were present near the germ and aleurone. The eroded endosperm contained partly digested starch granules but lost protein bodies, which resulted in the formation of small vacant spaces among the starch granules. Presumably, the spaces permitted further fungal invasion into the endosperm. Bechtel and Pomeranz (1978) showed that starch granules are surrounded by numerous protein bodies in the subaleurone region of the endosperm. It seems that rigid and tight structures of rice endosperm tissue prevent entry of the fungi at 85% rh (at 28°C), but the production of enzymes that degrade the tissue accompanied by active fungal growth enable penetration at 90% rh. The invading mycelia still remained in the milled rice kernels made from the infected brown rice stored at 90% rh. They were conspicuously present in the endosperm near the germ. These mycelia were also found in the milled rice derived from the *A. flavus*-infected rice that had been stored at 85% rh for 60 days but were rarely observed in those from *P. citrinum*-infected kernels kept under the same conditions.

Yield of Milled Rice

Table II shows the yields of milled rice derived from *A. flavus*-infected brown rice after storage. The yields decreased gradually during storage under both conditions. The yield after storage at 90% rh was lower than that at 85% rh, and lowest (79.6%) at the end of the 15-day storage period. The milling yield results correlated negatively to those of the fungal growth and invasion in the brown rice kernels, which were estimated from our scanning electron microscopy observation. The yields derived from *P. citrinum*-infected brown rice stored at 85% rh somewhat fluctuated and were in the range of 72.0–83.5% (data not shown), but pronounced changes in yield were not found in the rice stored at 90% rh (Table III).

TABLE II
Milling Yield and Content of Aflatoxins in Milled Rice and Rice Bran Plus Polish Fraction Made from *Aspergillus flavus*-Infected Brown Rice Stored at Two Humidity Levels at 28°C

Incubation Period (days)	Milling Yield[a] (%)	Fraction	Weight[b] (g)	Concentration of Aflatoxins (ng/g)		B1:G1 Ratio
				B1	G1	
Stored at 85% rh						
0	...	...	...	0	0	...
15	91.3	Milled rice	12.6	0	0	...
		Rice bran	1.2	0	0	...
30	88.4	Milled rice	12.2	0	0	
		Rice bran	1.6	40	100	0.4
45	85.4	Milled rice	12.3	0	0	
		Rice bran	2.1	240	800	0.3
60	83.3	Milled rice	12.0	20	40	
		Rice bran	2.2	2,620	5,310	0.5
Stored at 90% rh						
0	...	...	...	0	0	
5	89.5	Milled rice	11.9	190	160	
		Rice bran	1.4	1,860	1,800	1.0
10	89.7	Milled rice	11.3	300	160	
		Rice bran	1.3	6,640	4,240	1.6
15	79.6	Milled rice	10.9	250	160	
		Rice bran	2.6	8,930	6,530	1.4

[a]Milling for 6 min
[b]Wet basis.

TABLE III
Milling Yield and Citrinin Content in Milled Rice and Rice Bran Plus Polish Fraction from *Penicillium citrinum*-Infected Brown Rice Stored at About 90% rh and 28°C

Incubation Period (Days)	Milling Yield[a] (%)	Fraction	Weight[b] (g)	Concentration Citrinin (μg/g)
0	...	...	...	0
15	86.5	Milled rice	11.5	5.3
		Rice bran	1.8	8.2
30	82.0	Milled rice	10.9	11.3
		Rice bran	2.4	61.5
45	85.6	Milled rice	11.3	8.5
		Rice bran	1.9	101.4
60	82.7	Milled rice	11.0	4.3
		Rice bran	2.3	363.5

[a]Milling for 6 min.
[b]Wet basis.

Distribution of Aflatoxins and Citrinin

Table II shows the aflatoxin B1 and G2 contents in the milled rice and rice bran plus polish fractions made from *A. flavus*-infected brown rice stored at 85% rh for 60 days. The fungus produced significant amounts of toxins in this brown rice sample. Similar results were obtained from the milled rice stored under the same conditions (data not shown). Boller and Schroeder (1974) also reported that *A. parasiticus* rapidly invaded rough rice stored at 85% rh, the minimum humidity level for the production of aflatoxin. The content of the toxins in the brown rice increased over 30 days of storage and were maximal (B1, 2,640 ng/g; G1, 5,350 ng/g) at the end of the storage. Our scanning electron microscopic observation showed the fungus penetrated the germ and the aleurone in 30 to 45 days. Most of the toxins produced in the brown rice were removed in the milling process, except that the sample stored for 60 days and the rice milled from it still contained 4% of the toxins initially present in the brown rice. Table II shows the toxin contents of each fraction made from the *A. flavus*-infected rice that had been stored at 90% rh for 15 days. These results clearly showed that aflatoxin synthesis by the *A. flavus* group was much more stimulated at 90% than at 85% rh. Similar observations are reported elsewhere (Diener and Davis 1967, Tanaka et al 1984) for ground nuts and other nuts and seeds. About 10% of all toxins were present in the milled rice at the end of the storage. Table II also shows that humidity affected the toxin elaboration of aflatoxin B1 to G1 in the rice; the ratio was 1:4 in the brown rice stored at 85% rh, but more than 1.0 at 90% rh. Those obtained from milled rice stored at 85% rh were in the range of 0.3–0.1 during storage (data not shown). The formation of aflatoxin B1 by the fungus was more pronounced at 90% than at 85% rh. Diener and Davis (1967) reported that the ratio varied with temperature conditions in peanut culture.

Detectable amounts of citrinin were not observed in the milled rice and rice bran plus polish fraction derived from the *P. citrinum*-infected brown rice that had been stored at 85% rh for 90 days (data not shown). The rice samples had no appreciable changes in moisture content during the storage shown in Table I. In contrast, Table III shows that the fungus synthesized a considerable amount of citrinin in the brown rice at 90% rh, and its content increased over 30–45 days and reached the summit at the end of storage (60 days). The formation of the toxin was greatly enhanced at 90% rh at 28°C, similar to the action of *A. flavus*, but the minimum humidity value for production of citrinin must be more than 85%. About one fourth of the toxin present in the original brown rice still remained in the milled rice made from the brown rice stored for 60 days. This value is much higher than the corresponding value (10%) for the *A. flavus*-infected brown rice stored under the same conditions. It may reflect differences in the mode of invasion and ability to destroy endosperm tissue between *A. flavus* and *P. citrinum*.

Our study demonstrated that the mycotoxins aflatoxin and citrinin in fungus-damaged brown rice kernels were concurrent with the invading mycelia that were present in the caryopsis coat, aleurone, germ, and outer starchy endosperm, and especially conspicuous around the germ. Neither toxin was completely eliminated in a milling process. These results were consistent with the findings in our previous work (Takahashi et al 1984) on brown rice naturally infected by *A. versicolor* and also with those reported for rough rice artificially inoculated with *A. flavus* (Schroeder et al 1968).

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