Grain Development and Aflatoxin B1 Accumulation in Preharvest Rice Inoculated with Aspergillus parasiticus

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ABSTRACT

A rice cultivar (Japonica type), Cheong-cheong, was planted in a rice paddy in Southern Korea to examine the effects of Aspergillus parasiticus infection on the development of the grains. The grain was inoculated with the fungal inoculum at the milk stage of development. Ripening rates, 100-grain weights and aflatoxin B1 accumulation were observed at harvest. An enzyme-linked immunosorbent assay (ELISA) and immunohistochemical staining were performed to detect and confirm aflatoxin B1 in the samples. When the rice grains were inoculated with A. parasiticus the ripening rates (number of fully matured grains per total number of grains in a panicle) were not significantly different from the control samples. Examination of 100-grain weights showed no significant difference between the groups. The edible portion of inoculated grain exhibited significantly higher levels of toxins than did the rice hulls, and the embryo contained a higher proportion of toxins than the endosperm (P < 0.05). The immunohistochemical staining revealed positive findings for the embryo of inoculated grains, but not for the control embryo.

Key words: Aflatoxin B1, Aspergillus parasiticus, rice, ELISA, immunohistochemical staining

Aflatoxins are a group of toxic secondary metabolites produced by Aspergillus flavus and A. parasiticus (1). Considerable importance has been attached to the presence of aflatoxins in foods and feeds because of their carcinogenic, mutagenic, and teratogenic nature (16). Aflatoxin B1, the most toxic compound in this series, commonly contaminates cereal grains throughout the world (17). The frequent contamination of aflatoxin B1 in agricultural products is a potential hazard to human and animal health (1).

Knowledge of the rate of aflatoxin production and accumulation is very important in developing control strategies for aflatoxin contamination. Detection of aflatoxin and other mycotoxins produced by the toxin-producing fungi in agricultural products including corn, cottonseed, and wheat before harvest has been investigated (3, 4, 6, 9, 10, 12, 18). These studies have observed heterogeneity of the distribution of infected kernels and a large variability in the aflatoxin levels of individual kernels. Several studies have identified an association between insect damage and aflatoxin contamination in corn (10, 18).

Although the natural occurrence of mycotoxins in rice has been reported (7, 8), little is known about the toxin production during the development of the grain. Throughout Asia rice is the main source of carbohydrates and one of the most important agricultural products. Furthermore, Carballo and Miguel (2) observed that all of the aflatoxin-producing strains from agricultural products produced aflatoxin B1 on cracked rice. They concluded that rice is an efficient medium for aflatoxin production. The purpose of this study was to investigate the accumulation of aflatoxin B1 in preharvest rice through the inoculation of A. parasiticus at the milk stage and its effect on grain development.

MATERIALS AND METHODS

Reagents

All organic and inorganic chemicals and organic solvents were reagent grade or better. Horseradish peroxidase (HRP, type VI), bovine serum albumin (BSA), Tween 20 and 80, 1-ethyl-3, 3-dimethylaminopropyl-carbodiimide (EDPC), 2-2'-amino-di-3-ethylbenz-thiazoline-6-sulfonate (ABTS), hydrogen peroxide, and aflatoxin B1 were purchased from Sigma Chemical Co. (St. Louis, MO). Aflatoxin antiserum and horseradish peroxidase conjugates were prepared as described previously (5, 7, 13, 14).

Inoculum preparation

Fungal inoculum was prepared from A. parasiticus ATCC 15517. The fungus was grown on potato-dextrose agar (Difco Laboratories, Detroit, MI) in Petri plates for 10 days at 30°C. Spores were washed from the surface of the agar with sterile distilled water containing 0.1% Tween 80. The concentration of dislodged spores was determined with a hemacytometer and diluted to 106 conidia per ml. Spore suspensions were prepared the day before inoculation and stored at 4°C.

Rice cultivar and field experiment

A rice cultivar (Japonica type), Cheong-cheong, was chosen for this study. The cultivar was planted late in May in a large rice paddy field measuring one-third hectare in the Kyungnam Province of Southern Korea. The whole field was randomly divided into 8...
blocks. Five random samples from each of the 8 plots (4 control and 4 experimental) were obtained. All of the cultivation methods followed the recommendations of the Rural Development Administration of Korea (15).

The experimental groups were inoculated with the fungal spores at the milk stage. One milliliter of inoculum suspension was painted onto the grains with a small artist’s brush. This was accomplished when the weather was cloudy and calm.

**Harvest and sample preparation**

The cultivar usually reaches maturity 110 to 115 days after planting. Rice plants were harvested 110 days after planting and 35 days after inoculation. All the panicles selected were harvested by hand in the middle of September. They were dried in the field under the natural condition, and ripening rate (number of fully matured grains per total number of grains in a panicle) and 100-grain weights were determined. One-hundred mature grains were randomly selected for the measurement of grain weight.

From each of the 8 plots, 5 randomly selected areas were harvested and the grain was hulled by hand to make the samples for analysis. Once the hull was removed the brown rice was then separated into embryo (germ), endosperm, and bran (which is the portion between the hull and the endosperm). The final moisture levels of the brown rice after being dried in an oven at 105°C for 48 h were consistently below 12% (10.7 to 11.3%).

Each sample was divided into two parts; one part was used for immunohistochemical staining to confirm the presence of aflatoxin B₁ in samples, and the other was ground in a high-speed vibrating sample mill (Heiko, TI 200, Japan) to a fine powder (to pass a no. 200 sieve). The ground sample was used for the aflatoxin assay. Five grams of the sample were blended with 25 ml of 70% methanol (vol/vol) solution for 5 min at high speed (3,000 rpm) in a homogenizer (Nihonseiki Kaisha, AM-11, Japan). The extract was filtered through Whatman no. 4 filter paper and the filtrate was used directly for the aflatoxin ELISA.

**Aflatoxin analysis**

Quantities of aflatoxin B₁ in the samples were determined by ELISA. The procedure used for the ELISA was identical to those reported in detail previously (5, 13, 14, 17). Briefly, wells of polystyrene microtiter plates (Dynatech Laboratory, Alexandria, VA) were coated with specific aflatoxin antisera. Aflatoxin standard (in extracting solvent) or sample extract was mixed with an equal volume of aflatoxin-horseradish peroxidase conjugate. One-hundred µl of the mixture was added to the antibody-coated wells and the plate was incubated for 30 min at 37°C. The wells were washed and 100 µl of substrate solution was added and the plate was incubated again for 30 min at 37°C. Bound aflatoxin-horseradish peroxidase was determined on an ELISA reader. Aflatoxin content was calculated from a standard competition curve comparing the log of aflatoxin concentration versus absorbance at 405 nm.

**Immunohistochemical staining**

To determine the immunohistochemical localization, samples (embryo and endosperm of rice) were fixed for 12 h in 4% neutral buffered paraformaldehyde (pH 7.2) and embedded with O.C.T. compound (Zymed Laboratories, Inc., San Francisco, CA). Sections of 10 µm were prepared using a cryostat at −20°C and immunohistochemically stained using the method developed by Miller et al. (11). Sample sections were hydrated in distilled water and washed with 0.02 M phosphate-buffered saline (PBS). The washed sections were treated with 0.3% bovine serum albumin to reduce the nonspecific background staining. The primary antibody (mouse-derived anti-aflatoxin) was diluted 1:100 in 0.02 M PBS and was applied over the sections which were then incubated for 2 h at room temperature. Then the sections were washed three times in PBS (15 min, each) and incubated with the goat anti-mouse IgG-gold conjugates (5 nm) for 1 h. The sections were rinsed three times with PBS and deionized water and then incubated for 3 min with 100 µl of a mixture of initiating solution and enhancing solution of Silversearch-LM kit (Zymed Lab. Inc. San Francisco, CA). The sections were finally washed with PBS and examined using a microscope (Nikon, HFX-II, Japan).

**Data analysis**

The data from samples were compared by means of the Student t-test. The results are given in Table 1.

**RESULTS AND DISCUSSION**

There was no significant difference between the control and experimental groups in ripening rates and 100-grain weights (Table 1). Hart et al. (4), using a different experimental design, were able to demonstrate significant effects of fungus contamination on grain. When wheat grain was inoculated with Gibberella zeae before kernel maturity, grain weight was lower compared to that of wheat inoculated after maturity. In their work, however, the heads of wheat were sprayed with inoculum suspension and then covered with plastic bags for several hours. Had the authors used the design of Hart et al. we believe that a significant difference between the control and experimental groups would have been evident.

The unhulled rice grain, commonly known as rough rice, is composed of hull and brown rice. The mature brown rice is approximately 93% endosperm, 4% embryo, and the remaining 3% is bran, which is usually removed when rice is polished. The levels of toxin for each portion were analyzed and are given in Table 2. Table 2 shows toxin levels for the experimental and control groups. All of the 20 samples from the inoculated panicles contained aflatoxin B₁, but no grains from the control group had detectable toxin. Although the toxin was not detected in the control samples the reader is reminded that levels of less than 1 ng/ml are not measurable at this time.

Ram et al. (14) suggested that peanut butter extract interfered with the ELISA. They confirmed the matrix effects of commercial peanut butter on the results of the ELISA by analyzing the samples with high-performance liquid chromatography. They (13) also reported that there was negligible interference by corn and cottonseed extract. In this study, potential interference with the ELISA by the rice extract was tested by comparing the standard curve prepared in extraction solvent and in an aflatoxin-free rice extract (Figure 1). Standard curves resulting from the

**TABLE 1.** Grain development of rice inoculated with a spore suspension of A. parasiticus on grains at the milk stage

<table>
<thead>
<tr>
<th>Group</th>
<th>Grain ripening rate (%) (no. mature/ no. in panicle)</th>
<th>100-grain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, not inoculated</td>
<td>88.1 ± 1.4*</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Experimental, inoculated</td>
<td>85.9 ± 1.7</td>
<td>2.6 ± 0.1</td>
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</tbody>
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*All values represent mean ± SE of 20 samples.*
TABLE 2. Aflatoxin B₁ level in grain and each component of the rice inoculated with a spore suspension of A. parasiticus on grain at the milk stage

<table>
<thead>
<tr>
<th>Group</th>
<th>Unhulled rice</th>
<th>Rice hull</th>
<th>Brown rice</th>
<th>Embryo</th>
<th>Endosperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, not inoculated</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Experimental, inoculated</td>
<td>380.6 ± 65.9</td>
<td>18.7 ± 2.6</td>
<td>95.4 ± 16.5</td>
<td>26.1 ± 4.5</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

* ND, not detected; based on a standard curve, the detection limit was 1 ng/ml.

extraction solvent and the extract of rice were similar, showing negligible interference by rice in the ELISA performance. The linear response range of the standard curve in extraction solvent was between 1 and 100 ng/ml.

A large standard error of the mean was observed in unhulled rice as seen in Table 2. Variation also existed in toxin content when unhulled rice samples were divided into the other four components (rice hull and brown rice, which is composed of embryo, endosperm, and bran). Significantly higher levels of toxin were found in brown rice (the edible portion for humans) than in the rice hull \( (P < 0.05) \). In addition, higher levels of toxin were observed in the embryo than in the endosperm \( (P < 0.05) \). The presence of toxin in the edible portion of the inoculated rice indicates the possibility of fungal infiltration. Although we did not check the bran because it is usually removed when the rice is polished, we suspect that it also contains toxin to some degree. Aflatoxin formation occurs after the grains have been exposed to the fungal inoculum. This study shows that aflatoxin can be formed in inoculated rice during growth, and the toxin may accumulate in the embryo.

To confirm these findings, a procedure of immunohistochemical staining was performed. No remarkable appearance was found in the endosperm. However, the embryo from inoculated grains clearly showed a black color, which indicates the presence of toxin (Figure 2). This provides additional evidence of fungal infiltration. It also shows that toxin formation is maximized in the embryo.

There may be several pathways of fungal entry into the interior of the rice. We suspect environmental factors such as rainfall and temperature may encourage contamination. There was 24.08 cm of rainfall between the time of inoculation and harvest. No attempts were made to evaluate whether the rainfall was favorable or unfavorable for the fungal infiltration. The average temperature between the

FIGURE 1. Effect of rice extract on the standard curve for aflatoxin B₁ as determined by ELISA. O—O Extraction solvent, O--O aflatoxin-free rice extract.

FIGURE 2. Immunohistochemical staining of rice embryo with anti-mouse aflatoxin antibody. Sections show the plumule (p), which will be a primary leaf during development, and the coleoptile (c). Embryo with accumulated aflatoxin clearly shows positive results that appear to be black. A, control embryo (not inoculated); B, embryo of experimental group (inoculated with A. parasiticus); ×100.
time of inoculation and harvest was over 25°C (21.4 to 28.3°C). However, it was found that temperatures above 25°C were less favorable for infection of G. zeae in wheat (4).

Although a possible mode of entry in corn and cotton-seed is through an opening made by insects (3, 10, 18), it is not obvious to us how the fungus could enter the interior of the rice when the grains were inoculated. In related work we have studied toxin accumulation in preharvest rice by three inoculation methods using the fungal spore suspension. It was observed that much more toxins were detected in unhulled rice when inoculations were made by needle or knife than by using a brush (5). Therefore, further research on the production of aflatoxin in rice grains should be conducted in order to ascertain the mechanism of the fungal infiltration and aflatoxin accumulation in the embryo under a controlled environment.

Results obtained in this experiment revealed two major points: A. parasiticus can enter the interior of the rice grain on contact; and although aflatoxin was identified in all parts of the grain in the experimental group, higher toxin levels were found in the embryo than in the endosperm.

Since rice is one of the major foods for Asian countries our results should be studied and examined further. We also have to remember that rice hulls and bran, the residues after polishing, are used as feed for domestic animals, which in turn are a source of protein for humans. It is possible that A. parasiticus contamination can be spread through this process.

REFERENCES


