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Variation of Aflatoxin B_1 Production in Brown Rice Inoculated with *Aspergillus parasiticus* under Different Storage Conditions

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현미의 저장조건에 따른 aflatoxin B_1 생성의 변화

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ABSTRACT—A rice cultivar (Japonica type), *Cheong-cheong*, was used to examine the ability as a substrate for aflatoxin production. Brown rice samples were inoculated with *Aspergillus parasiticus*, stored at various conditions, and observed the production of aflatoxin B_1 during storage. Enzyme-linked immunosorbent assay (ELISA) was performed to detect aflatoxin B_1 in the samples. A temperature of 28°C favored the aflatoxin production in the samples. Remoisturizing brown rice to 15.8% encouraged the fungus to produce the aflatoxin significantly ($p<0.05$). Brown rice was a more efficient medium for the aflatoxin production than the whole rice grain, but autoclaved brown rice was the most efficient medium. Significantly more aflatoxin was detected in brown rice when it was incubated at room temperature for 3 months than that incubated for 15 days ($p<0.05$). This study showed that temperature and moisture content were factors affecting aflatoxin B_1 production in rice, and also indicated that other factors such as husking and storage periods were also risk determinants. This study provided evidence that rice could be an efficient medium for aflatoxin production.

Key words □ aflatoxin B_1, *A. parasiticus*, rice, storage

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. Aflatoxin B_1, the most toxic compound in this series, commonly contaminates cereal grains throughout the world.[2,3] The frequent contamination of aflatoxin B_1 in agricultural products is a potential hazard to human and animal health.[1]

Information concerning the rate of aflatoxin production and accumulation is very important in developing control strategies for aflatoxin contamination. The detection of aflatoxin and other mycotoxins produced by toxin-producing fungi in agricultural products including corn, peanut butter and cottonseed has been widely investigated.[3-7]

Although the natural occurrence of mycotoxins in rice[8,9] and aflatoxin accumulation in preharvested rice[10] have been reported, little is known about toxin production during the storage phase. Mold growth resulting in postharvest contamination by toxins is more likely to occur when grains are stored at marginally safe moistures and temperature levels. Additionally, in the storage environment substrate suitability may affect the level of toxin production. Throughout Asia rice is the main source of carbohydrates and one of the most important agricultural products. Carballo and Miguel[11] observed that all of the aflatoxin-producing strains in the *A. flavus* group isolated from agricultural products produced aflatoxin B_1 on cracked rice. They suggested that rice is an efficient medium for aflatoxin production by *A. flavus*. The purpose of this study is to investigate whether rice is an efficient substrate for aflatoxin production by confirming the ability of *A. parasiticus* to produce aflatoxin B_1 in rice cul-

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tures under various storage conditions after harvesting.

MATERIALS AND METHODS

Reagents

All 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-dimethyl-amino-propyl-carbodiimide (EDPC), 2-2'-amino-di-3-ethylbenzthiazoline-6-sulfonate (ABTS), hydrogen peroxide and aflatoxin B_1 were purchased from Sigma Chemical Co. (St. Louis, MO). Aflatoxin antisera and horseradish peroxidase conjugates were prepared as previously described.[5,6,8]

Inoculum preparation

Fungal inoculum was prepared from single-spore cultures of *A. parasiticus* ATCC 15517. The fungus was grown on potato-dextrose agar (Difco Lab., Detroit, MI) in Petri plates for 10 days at 30°C. Spores were washed from the plates with sterile distilled water containing 0.1% Tween 80. The concentration of dislodged spores was determined with a hemacytometer and diluted to 10^6 conidia/m*l*. Spore suspensions were prepared one day before inoculation and stored at 4°C.

Rice and aflatoxin production

A rice cultivar (Japonica type), *Cheong-cheong*, was planted in a rice paddy field in Southern Korea in May (12). Panicles of the rice were harvested from the rice paddy in September and dried under natural conditions. Several hundred mature whole grains were randomly selected and husked by hand and later separated into hull and hulled rice (brown rice).

Five grams of each sample were put in a test tube and inoculated with 100 μ*l* of the *A. parasiticus* spore suspension and then incubated at various conditions. In the first treatment, brown rice samples were divided into three temperature groups: incubation at 28°C, room temperature (non-temperature controlled, below 20°C) and temperature cycling for 15 days. Temperature cycling was accomplished by using two incubators preset at 28±1°C and 15±1°C. Cycling was performed by manually shuttling samples between incubators at 16-hr and 8-hr intervals, respectively. Each 24-hour period was regarded as one temperature cycling (16 hrs of daylight at 28±1°C and 8 hrs of darkness at 15±1°C). The second experiment was to study the effect of moisture content on aflatoxin production. The fresh brown rice with 10.7% moisture content and brown rice remoistened to 15.8% were inoculated with the same amount of fungal inoculum and incubated at temperature cycling for 15 days. To remoisturize, 2.5 m*l* of distilled water was added to 5 g of each sample. The third experiment was used for aflatoxin production by sample type, i. e. whole rice grain, brown rice and autoclaved brown rice. Samples were incubated at temperature cycling for 15 days. The fourth experiment was aimed to investigate aflatoxin production in rice under different storage periods. Brown rice samples were stored at room temperature for 15 days and 3 months.

Sample preparation and aflatoxin analysis

After incubation, the rice samples were ground in a high speed vibrating sample mill (Heiko, TI 200, Japan) to a fine powder (to pass No. 20 sieve) which was used for aflatoxin assay. A 5-g of the sample was blended with 25 m*l* of 70% methanol (v/v) for 5 minutes at high speed (3,000 rpm) in a homogenizer (Nihonseiki Kaisha, AM-11, Japan). The extract was filtered through a layer of Whatman No. 4 filter paper and the filtrate was used directly for aflatoxin B_1 analysis by ELISA, a procedure identical to those reported in detail previously.[3,5,6,10,13] Briefly, wells of polystyrene microtiter plates (Dynatech Lab., 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 μ*l* 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 μ*l* 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 concentrations vs. absorbance at 405 nm.

Data analysis

The data from samples were compared by means of the Student *t-test* or the analysis of variance (ANOVA). Significant differences among means were determined by using Duncans' multiple range test.

RESULTS AND DISCUSSION

Effects of rice extract on ELISA standard curve

The whole rice grain, commonly known as rough rice, is composed of the hull and hulled rice (brown rice). The mature brown rice is approximately 93% endosperm, 4% embryo, and the remaining 3% is bran, which is usually removed when the rice is polished. Neither fresh whole grains nor brown rice had detectable aflatoxin B_1 (1 ng<m*l*). We tested for potential interference with the ELISA by the rice extract by comparing the standard curve prepared in extraction solvent and an aflatoxin-free rice extract.[10] These curves were similar, showing negligible interference by rice in ELISA performance. Linear response range of the standard curve in extraction solvent was between 1 and 100 ng/m*l*.

Effects of temperature on aflatoxin production

Brown rice samples subjected to a constant temperature of 28°C significantly increased the production of aflatoxin B_1 (4.3 mg/kg), as compared with those held at cycling between 28°C and 15°C (3.4 mg/kg) ($p<0.05$) (Table 1). There was no significant difference between the aflatoxin B_1 level in the samples incubated at the constant 28°C and those at room temperature (3.9 mg/kg) or between the room temperature and cycling samples, but there was a significant difference between cycling and constant 28°C samples. Therefore constant temperature of 28°C favored aflatoxin B_1 production in rice medium.

Temperature is one of the most important environmental factors influencing the growth and toxin production of toxigenic fungi. In case of *A. parasiticus*, the optimum temperature for growth is 35°C but it has been reported that maximum aflatoxin biosynthesis occurs at 25~30°C on both synthetic and natural media in laboratory experiments.[2] Schindler[14] reported that *A. parasiticus* will not produce aflatoxins outside 7.5°C~40°C range when grown on wort agar or other agricultural commodities. Trucksess *et al.*[15] indicated that a temperature of 16°C was generally too low for aflatoxin production by either the inoculated or naturally occurring strains of *Aspergillus*.

The results of our study were contrasted to that of Lin *et al.*[16] They found that the cycling of temperature between 33°C and 25°C for 14 days increased aflatoxin accumulation (5,247~5,898 μg/25 m*l*) by *A. parasiticus* in yeast-extract sucrose (YES) medium more than a constant temperature of 25°C (2,060 μg/25 m*l*) or 33°C (5,003 μg/25 m*l*). They also observed that whenever temperature programs included higher temperatures (33°C), aflatoxin B_1 production or accumulation was favored, and when they included lower temperatures (25°C) favored aflatoxin G_1 production or accumulation. We started the experiment after harvesting the rice at the end of September. Air temperature during this season is below 20°C, so the rice in the room temperature program had been stored in cool weather. This may inhibit the fungus from producing the toxin. If the authors had performed the experiment in the summer season we suspect that a significant higher production of toxin in room temperature group would have been evident. In the summer season the average air temperature is usually over 25°C in Korea.

Effects of moisture content on aflatoxin production

A. parasiticus survived in both the fresh and remoistened brown rice and was capable of being toxigenic. As shown in Table 2, remoistened rice produced as much as 2.1 times

Table 1. Aflatoxin B_1 production in brown rice inoculated with *A. parasiticus* after 15 days under different temperature conditions

Storage temperature	Aflatoxin B_1 (mg/kg)
28°C	4.3 ± 0.6^{a}
Cycling[1]	3.4 ± 0.4^{b}
Room temperature	3.9 ± 0.3^{ab}

[1] 28°C for 16 hours, 15°C for 8 hours
All values represent mean±S.E. of 20 samples.
Values in the same column followed by different superscript letters are significantly different ($p<0.05$).

Table 2. Aflatoxin B_1 production in brown rice inoculated with *A. parasiticus* after 15 days with different moisture content[1]

Moisture content	Aflatoxin B_1 (mg/kg)
N.C[2]	3.4 ± 0.5^{b}
T.C[3]	7.3 ± 1.2^{a}

[1] Samples inoculated with *A. parasiticus* were stored at 28°C for 16 hours and 15°C for 8 hours.
[2] Natural condition (10.7% of moisture content).
[3] Treated condition: 2.5 m*l* of distilled water was added to 5 g of rice in test tube (15.8% of moisture content).
All values represent mean±S.E. of 20 samples.
Values in the same column followed by different superscript letters are significantly different ($p<0.05$).

more aflatoxin B_1 (7.3 mg/kg) than fresh rice (3.4 mg/kg) ($p<0.05$). This result provided evidence that the moisture level of rice is a factor in aflatoxin production.

Wilson *et al.*[16] found that *A. flavus* survived better in 19.6 % remoistened corn than in the freshly harvested corn with 29.4% moisture when the samples were incubated at 27°C. Moisture content and water activity (a_w) as well as temperature are generally considered as critical factors for mold growth. *A. flavus* does not grow on corn if the moisture content is at or below 17.5% (which equates with an a_w of about 0.79) and for aflatoxin production in corn at 30°C an $a_w>0.86$ is necessary.[17] Trucksess *et al.*[15] translated measured moisture to a_w on the basis that the gravimetric determination of moisture was more precise than the direct measurement of a_w. In their study limiting a_w values for growth of *A. flavus* at 26°C and 32°C were 0.73, 0.69 and 0.75 for corn, soybeans and pinto beans, respectively.

Effects of sample type on aflatoxin production

Three conditions of rice (whole grain, brown rice and autoclaved brown rice) were compared in their susceptibility to aflatoxin production (Table 3). We suspect that the infection of *A. parasiticus* is dramatically different in whole grain, brown rice and autoclaved brown rice. Although little growth of the fungus was apparent in the whole grain samples, some toxin was detected.

Rice is one of the major staple for Asian countries. The traditional method of storing rice in Korea is to store the rice as whole grain. The result of our study demonstrates that husking can be a risk factor and whole grain is more resistant than hulled rice to the production of aflatoxin during storage.

Table 3. Aflatoxin B_1 production in rice inoculated with *A. parasiticus* after 15 days by different sample type[1)]

Sample type	Aflatoxin B_1 (mg/kg)
Whole rice grain (unhulled rice)	0.1 ± 0.1^c
Brown rice (hulled rice)	3.4 ± 0.1^b
Brown rice, autoclaved	56.4 ± 1.9^a

[1)] Samples inoculated with *A. parasiticus* were stored at 28°C for 16 hours and 15°C for 8 hours.
All values represent mean±S.E of 20 samples.
Values in the same column followed by different superscript letters are significantly different ($p<0.05$).

Autoclaving, similar to cooking the rice, encouraged the fungus to produce the toxin. A high level of aflatoxin (56.4 mg/kg) was detected in autoclaved brown rice. It may be the result of elimination of competing fungi contaminating the harvested rice. Or it could be due to breakdown of starch to simple sugars during autoclaving. It is almost the same level of aflatoxin production as when Kim[18] used a yeast-extract sucrose (YES) broth medium. Lin *et al.*[19] also found that 2,060 μg/25 m*l* of aflatoxin B_1 was produced when *A. parasiticus* NRRL 2999 was inoculated on the medium and incubated at 25°C. These data indicate that rice could be an efficient substrate for aflatoxin production as observed by Carballo and Miguel.[11]

Effects of storage periods on aflatoxin production

Variation of aflatoxin production in rice due to duration of storage was observed as shown in Table 4. When brown rice was incubated for 3 months at room temperature, much more aflatoxin B_1 (62.8 mg/kg) was produced than when incubated for 15 days (3.9 mg/kg). It may be due to accumulation of toxin over a period of time, although we did not measure the rate of change of toxin production with time. In related work we have studied toxin accumulation in brown rice and autoclaved brown rice. It was observed that a little more toxins were detected in autoclaved brown rice than in brown rice in 3 months.[13] Rice starch is probably being broken to provide adequate substrate for toxin production in 3 months. Therefore, further research on examining the linearity of such a change should be conducted under a controlled environment.

Kim and Lee[10] reported that a residue level (μg/kg) of aflatoxin B_1 was produced in preharvest rice inoculated with *A. parasiticus*. We suspect that aflatoxin production in

Table 4. Aflatoxin B_1 production in brown rice inoculated with *A. parasiticus* under different storage periods[1)]

Storage periods	Aflatoxin B_1 (mg/kg)
15 days	3.9 ± 0.3^b
3 months	62.8 ± 2.8^a

[1)] Samples inoculated with A. parasiticus were stored at room temperature (non-temperature controlled, below 20°C).
All values represent mean±S.E. of 20 samples.
Values in the same column followed by different superscript letters are significantly different ($p<0.05$).

rice mainly occurs during the storage phase as found in other agricultural commodities such as corn and peanuts. Humans have to focus their efforts on preventing the toxigenic fungi before harvesting and reducing favorable conditions for toxin production during storage.

The difference in the rate of production of aflatoxin B_1 in rice during the storage phase was related to temperature, moisture content, sample type (whole grain, brown rice and autoclaved brown rice) and duration of storage. Although it appears that the major contamination of rice with aflatoxigenic fungus occurs before or during harvest, storage condition can increase the production of aflatoxin B_1. We have to remember that aflatoxin can be produced even in whole grain if it is contaminated with the toxigenic fungus. We also have to remember that rice hull and bran, the residue after husking and polishing, are used as feed for domestic animals which in turn are source of protein for humans.

This study showed that *A. parasiticus* also produced the most hazardous aflatoxin, i.e. aflatoxin B_1 in rice samples incubated in different temperature regimes and humidity. Carballo and Miguel[11] observed that cracked rice was an efficient medium for aflatoxin production by *A. flavus*. Therefore, we suggest that rice could be another substrate for aflatoxin production.

국문요약

쌀에 대하여 aflatoxin 생성을 위한 기질로서의 능력을 알아보기 위하여 현미(청청벼) 시료에 *Aspergillus parasiticus*를 접종하고 조건을 달리하여 저장하면서 aflatoxin B_1의 생성을 관찰하였다. 시료중의 aflatoxin B_1의 분석은 ELISA를 이용하여 수행하였다. 현미 시료에서 aflatoxin B_1 생성에 가장 좋은 온도는 28°C였으며, 시료의 수분함량을 15.8%로 증가시킨 경우 aflatoxin B_1의 생성이 유의하게 증가하였고 ($p<0.05$), 고압증기멸균시킨 시료는 aflatoxin B_1 생성에 보다 효과적인 기질이 되었다. 실온에서 3개월 동안 저장한 현미에서는 15일 동안 저장한 경우에 비하여 aflatoxin B_1 생성이 유의한 증가를 보였다($p<0.05$). 따라서 저장온도 및 수분함량이 쌀에서도 aflatoxin B_1 생성에 영향을 미치는 바를 나타내었으며, 또 시료의 상태 및 저장기간도 쌀에서 aflatoxin 생성 위험요인으로 작용할 수 있음이 제시되었다. 이러한 결과로부터 쌀이 aflatoxin 생성에 좋은 기질이 될 수 있는 것으로 평가된다.

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