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Behaviour of *Aspergillus flavus* and *Fusarium graminearum* on rice as affected by degree of milling, temperature, and relative humidity during storage

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ABSTRACT

We investigated the survival and growth patterns of *Aspergillus flavus* and *Fusarium graminearum*, as well as mycotoxin production, on Korean rice as affected by the degree of milling (rough, brown, and white rice) and storage conditions (21 °C/85% relative humidity [RH], 21 °C/97% RH, and 30 °C/85% RH). When rice was stored at 21 °C/85% RH, the population of *A. flavus* remained constant and aflatoxin was not produced, regardless of the degree of milling. At 21 °C/97% RH and 30 °C/85% RH, the populations of *A. flavus* increased significantly ($P \leq 0.05$) and aflatoxins were produced. The highest population of *A. flavus* and highest amount of aflatoxin B_1 were observed on brown rice stored at 21 °C/97% RH. For *F. graminearum*, when stored at 85% RH, the populations were reduced to less than a detectable level (5 CFU/g of rice) within 120 days and no deoxynivalenol (DON) was produced, regardless of the degree of milling and storage temperature. However, at 21 °C/97% RH, the population of *F. graminearum* increased significantly ($P \leq 0.05$) and DON was produced on all types of rice. Findings from this study provide insights concerning storage conditions necessary to prevent growth and mycotoxin production by *A. flavus* and *F. graminearum* on Korean rice with different degrees of milling.

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the global population (FAO, 2004a) and provides 20% of the dietary energy supply in the world (FAO, 2004b). According to the Food and Agriculture Organization of the United Nations, world rice production has increased steadily in recent years. Production increased from *ca.* 518 million tons in 1990 to *ca.* 599 million tons in 2000 and *ca.* 701 million tons in 2010 (FAOSTAT, 2013). Rice is stored for several months or even years as rough rice after harvesting, and brown rice or white rice after milling. Rough rice consists of the hull, bran, and endosperm. Rough rice is dehulled to produce brown rice and the bran layer of brown rice is removed to produce white rice (Skyrme et al., 1998). Rice is usually consumed in the latter form, but demands for brown rice have increased because of its high nutritional value (FAO, 2004b; Heinemann et al., 2005).

As rice is an essential part of the human diet of many people, it is important to maintain its sensorial, nutritional, and microbiological qualities. The growth of some fungal species on rice, with consequent mycotoxin production, results in a microbiological safety concern (Kumar et al., 2008; Reddy et al., 2009). The major genera of fungi found on rice are *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Mucor*, *Rhizopus*, *Trichoderma*, *Curvularia*, *Helminthosporium*, and *Cladosporium* (Makun et al., 2007). Among them, *Aspergillus flavus* and *Fusarium graminearum* raise particular concern because some strains can produce mycotoxins. Contamination of rice with mycotoxigenic fungi and the presence of mycotoxins have been reported in several countries (Desjardins et al., 1997; Fredlund et al., 2009; Makun et al., 2007; Ok et al., 2009; Tanaka et al., 1988). Fredlund et al. (2009) reported that 21% of rice samples collected from Swedish retail markets were contaminated with *A. flavus*. Total aflatoxin in contaminated rice was as high as 50.7 μg/kg Park

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et al. (2005) reported that 17% and 10% of polished rice samples obtained from grain wholesale markets in the Republic of Korea were contaminated with *A. flavus* and *F. graminearum*, respectively; the mean aflatoxin B_1 (AFB_1) and deoxynivalenol (DON) concentrations in rice were 4.3 and 139 ng/g, respectively.

A. flavus is a mycotoxigenic fungus that produces AFB_1 and aflatoxin B_2 (AFB_2). AFB_1 is classified as an International Agency for Research on Cancer (IARC) Group 1 human carcinogen (IARC, 1993). The optimum temperatures for growth and aflatoxin production are *ca.* 33 °C and 16–31 °C, respectively, and the optimum water activities (a_w) for growth and aflatoxin production are 0.98 and 0.95–0.99, respectively (ICMSF, 1996). An outbreak of 397 cases of hepatitis with 106 deaths occurred in India in 1974. It was concluded that this outbreak was associated with the consumption of maize heavily contaminated with *A. flavus* and containing aflatoxin of concentrations up to 15.6 μg/g (Krishnamachari et al., 1975). In Kenya, 317 people became ill after eating maize contaminated with aflatoxin; 125 deaths occurred in this outbreak (CDC, 2004). The government of the Republic of Korea has set limits of 15 ng/g and 10 ng/g for total aflatoxins and AFB_1, respectively, in cereal grains (Korean Ministry of Food and Drug Safety, 2013). In the USA, the limit for total aflatoxins in food is 20 ng/g, but no limit has been specifically set for AFB_1 (FAO, 2003). In the EU, maximum concentrations of 10 ng/g for total aflatoxins and 5 ng/g for AFB_1 have been established in rice (European Commission, 2010).

F. graminearum is a plant pathogen that can produce DON, nivalenol, and zearalenone (Pitt and Hocking, 2009). Growth of *F. graminearum* is most rapid at 25 °C and a_w 0.95–0.995, and DON production is highest at 25 °C and a_w 0.995 (Ramirez et al., 2006). DON is classified by the IARC as a Group 3 agent (not classifiable as to carcinogenicity in humans) (IARC, 1993). However, consumption of food contaminated with DON may result in nausea, vomiting, gastrointestinal upset, dizziness, diarrhoea, and headache (JECFA, 2002). An outbreak has been reported in India in 1987, in which 97 people became sick after eating wheat bread contaminated with DON and other trichothecene mycotoxins (Bhat et al., 1989). The limit for DON in grains in the Republic of Korea is 1000 ng/g (Korean Ministry of Food and Drug Safety, 2013). In the USA, the limit of DON in finished wheat products for human consumption is 1000 ng/g (FDA, 2010). The EU has set limits for DON in unprocessed cereal (1250 ng/g) and in cereals for direct human consumption (750 ng/g) (European Commission, 2006).

Although there have been no documented outbreaks of foodborne intoxication associated with the consumption of rice containing aflatoxin or DON, there is a potential risk of mycotoxin production in rice contaminated with *A. flavus* or *F. graminearum* and stored at abusive temperature and relative humidity (RH) conditions. In countries such as the Republic of Korea, where rice is a staple food and the climate is changing from temperate to subtropical due to global warming, potential risks of human illness associated with consumption of rice and other grains containing mycotoxins are likely to increase. Mycotoxins are difficult to eliminate in foods without compromising sensorial and nutritional quality because they are heat-resistant and do not decompose readily (Korzun, 2002; Shapira and Paster, 2004). It is important to prevent contamination of rice with mycotoxigenic fungi and store rice under conditions that will prevent growth mycotoxin production. The growth of fungi and production of mycotoxins in rice and other grains are affected by the availability of nutrients and environmental conditions, such as temperature, a_w, and pH (Holmquist et al., 1983; Llorens et al., 2004; Mylona et al., 2012; Ramirez et al., 2006; Sweeney and Dobson, 1998).

There have been a number of reports describing conditions affecting growth of fungi and production of mycotoxins affected by storage temperature and a_w. However, none has addressed the patterns of fungal growth and mycotoxin production in rice as affected by different degrees of milling and long-term storage under various temperatures and RH. The study reported here was done to determine the patterns of growth and survival of *A. flavus* and *F. graminearum*, as well as mycotoxin production, on rice as affected by the degree of milling, temperature, RH, and storage time.

2. Materials and methods

2.1. A. flavus strains and preparation of inocula

Five strains of *A. flavus* known to produce AFB_1 and AFB_2 were used in this study: *A. flavus* strain ATCC 22546 (isolated from mouldy corn), CN 008 (isolated from malted wheat), CN 028 (isolated from malted wheat [*nuruk*]), CN 029 (isolated from malted wheat [*nuruk*]), and M 2034 (isolated from fermented soybeans [*meju*]). These strains were chosen because they had been isolated from grain-based products and their abilities to produce mycotoxin had been confirmed. *A. flavus* strain ATCC 22546 was obtained from Korean Culture Center of Microorganisms in Seoul, Republic of Korea, and strains CN 008, CN 028, CN 029, and M 2034 were supplied by Dr. Soo-Hyun Chung (Department of Food and Nutrition, Korea University, Seoul, Republic of Korea). Each strain was streaked separately onto potato dextrose agar (PDA; BBL/Difco, Sparks, MD, USA) supplemented with 10% tartaric acid (PDAT; pH 3.5) slants and incubated at 28 °C for 7–10 days. Spores were harvested by depositing 10 mL of distilled water (DW) containing 0.02% Tween 80 (Junsei, Tokyo, Japan) on the mat surface, gently rubbing with a sterile loop, and filtering the suspension through sterile cheesecloth. Spore counts (spores/mL) were determined using a haemocytometer. More than 98% of the propagules were spores. A five-strain cocktail of *A. flavus* spores was prepared by combining suspensions of each of the five strains (2×10^6 spore per strain). The suspension was centrifuged at 20,000× *g* for 15 min. The supernatant was decanted and spores were resuspended in sterile DW to give *ca.* 6.3 log spores/mL.

2.2. F. graminearum strains and preparation of inocula

Five strains of *F. graminearum* known to produce DON were used in this study: *F. graminearum* strains KACC 46434 (isolated from rice), KACC 46437 (isolated from barley), KACC 46438 (isolated from barley), KACC 46439 (isolated from barley), and KACC 46441 (isolated from rice). These strains were chosen because they had been isolated from grain and their abilities to produce DON had been confirmed. All strains were obtained from the Rural Development Administration-Genebank Information Center, Suwon, Republic of Korea. Each strain was inoculated separately on the PDAT plates and incubated at 28 °C for 7 days. Five agar plugs (*ca.* 6 mm diam.) from the edges of colonies of each strain were deposited in 100 mL of carboxymethyl cellulose (CMC) broth (ammonium nitrate [NH_4NO_3], 1 g; monopotassium phosphate [KH_2PO_4], 1 g; magnesium sulphate heptahydrate [$MgSO_4 \cdot 7H_2O$], 0.5 g; yeast extract, 1 g; carboxylmethyl cellulose, 15 g; DW, 1 L) and incubated on a rotary shaker at 200 rpm for 5 days at 25 °C. The CMC culture was filtered through sterile cheesecloth and spore counts for each strain were determined with a haemocytometer. More than 98% of the propagules were spores. A five-strain cocktail of *F. graminearum* spores was prepared by combining suspensions of each of the five strains (2×10^6 spore per strain). The suspension was centrifuged at 20,000× *g* for 15 min. The supernatant was decanted and the pellet was resuspended in sterile DW to give *ca.* 6.3 log spores/mL.