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ORIGINAL ARTICLE

Potential for aflatoxin B_1 and B_2 production by *Aspergillus flavus* strains isolated from rice samples

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Abstract In this study, we investigated the potential for aflatoxin B_1 (AFB_1) and B_2 (AFB_2) production in rice grain by 127 strains of *Aspergillus flavus* isolated from rice grains collected from China. These strains were inoculated onto rice grains and incubated at 28 °C for 21 days. AFB_1 and AFB_2 were extracted and quantified by high-performance liquid chromatography coupled with fluorescence detection. Among the tested strains, 37% produced AFB_1 and AFB_2 with levels ranging from 175 to 124 101 μg kg^{-1} for AFB_1 and from not detected to 10 329 μg kg^{-1} for AFB_2. The mean yields of these isolates were 5884 μg kg^{-1} for AFB_1 and 1968 μg kg^{-1} for AFB_2. Overall, most of the aflatoxigenic strains produced higher levels of AFB_1 than AFB_2 in rice. The obtained information is useful for assessing the risk of aflatoxin contamination in rice samples.

1. Introduction

Aflatoxins (AFs) are a group of mycotoxins produced as secondary metabolites by the spoilage of *Aspergillus* fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus* (Davis and Diener, 1983; Miguel and Guillermo, 1986; Yu et al., 2003; Klich, 2007). These fungi can grow on various agricultural commodities and generate aflatoxins before and during harvest, handling, shipment and storage (Peraica et al., 1999; Giray et al., 2007; Reddy et al., 2009a). The most important members are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). They are highly toxic and carcinogenic compounds that cause disease in livestock and humans (Richard, 2007). The International Agency for Research on Cancer (IARC) has clarified AFB_1, AFB_2, AFG_1 and AFG_2 in the group I as human carcinogens (IARC, 1993).

Rice (*Oryza sativa* L.) is one of the most important staple foods in the world. Especially in Asian countries, large amounts of rice are consumed per capita per year. According to the Food and Agriculture Organization (FAO), in 2008 the worldwide rice production is about 68 501.3 million tons. The main rice producing countries are China, India, Indonesia, Bangladesh, Myanmar, Thailand and Vietnam (http://www.fao.org/newsroom/common/ecg/1000820/en/Rmprod0308.pdf). Rice cultivation is usually conducted in subtropical environments, which are characteristically warm and humid. Rice is generally dried after harvesting. Due to inappropriate storage conditions, rice can be

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an ideal substrate for mycotoxin-producing fungi. In recent years, numerous studies have revealed high levels of aflatoxins and fungal contamination in rice in many countries (Tanaka et al., 2007; Reddy et al., 2008; Reddy et al., 2009a,b; Reiter et al., 2010; Aydin et al., 2011; Bansal et al., 2011; Hussaini et al., 2011; Almeida et al., 2012; Elena et al., 2013; Ok et al., 2014). However, mycotoxin-producing fungi are less commonly reported for rice than for many other cereal crops (Tanaka et al., 2007).

In China, rice is the most important cereal, the staple food for more than 65% of the population (Zhang et al., 2005), and it is the subsistence crop for the most resource-poor rice farmers and consumers in rural areas. China ranks first in total annual rice production and produced 29% of the world's rice in 2006 (www.fao.org). Some studies have reported that rice in China has been contaminated with mycotoxins such as aflatoxins and fumonisins (Tang, 1999; Trucksee, 2000; Liu et al., 2006; Wang and Liu, 2007; Sun et al., 2011; Lai et al., 2014). However, only limited data are available on the aflatoxigenic fungal contamination of rice in China and on the ability of local fungal strains to produce mycotoxins (Liu et al., 1981).

In our preliminary study, rice intended for human consumption in China in 2009–2011 was extensively collected from different geographical areas and a number of *A. flavus* strains have been isolated from the rice samples. The aim of this study was to evaluate the ability of *A. flavus* strains to produce aflatoxins on rice grains in vitro.

2. Materials and methods

2.1. Strains and mycotoxin production

A total of 127 strains of *A. flavus*, which were isolated from rice samples collected from twelve provinces in China, were selected to test for aflatoxin production in cultures of rice grain. Spore suspensions of the individual *A. flavus* strains were prepared by growing the fungi on Petri dishes for 5 days with potato dextrose agar. After incubation at 25 °C, spores were harvested by adding sterilized distilled (10 mL for each plate) water on each plate. The spore suspension thus obtained was filtered using cheesecloth, and spores were counted using a hemocytometer and brought to a final concentration of 10^7 conidia per mL. All treatments were replicated two times.

2.2. Rice grain cultures

Twenty-five grams of polished rice grain was placed in Erlenmeyer flasks (150 mL), 25 mL of distilled water was added, and they were autoclaved at 121 °C for 1 h and allowed to stand overnight. Each flask was inoculated with 2 mL of the spore suspension described above, incubated at 28 ± 1 °C in dark/night of 12/12 h for 21 days and shaken once or twice daily for 3 days to aid in even distribution of the inocula. After incubation, the moldy rice grains were autoclaved at 100 °C for 30 min, then dried overnight at 60 °C and used for the extraction of AFB_1 and AFB_2.

2.3. Extraction of aflatoxins from moldy rice grains

Aflatoxins were extracted from moldy rice grains by the method described in our previous study (Lai et al., 2014) with minor modifications. The homogenized moldy rice samples (1.5 g, dry weight) were extracted with 6 mL of a 79/20/1 (v/v/v) mixture of MeCN/H_2O/AcOH in an ultrasonic cleaner for 15 min. After centrifugation for 5 min at 3000 rpm, the upper layer was filtered through a filter paper (Whatman No 44) and then processed by dispersive liquid–liquid microextraction (DLLME). Two hundred microliters of $CHCl_3$ was added to a 1 mL aliquot of 79% MeCN extract and was mixed with a vortex mixer for 30 s. The mixture was then rapidly injected into a 15 mL screw-cap glass centrifuge tube with a conical bottom that contained 5 mL of 2% NaCl solution (pH 3.0). This ternary component system was mixed for few seconds with a vortex mixer. After centrifugation for 5 min at 3000 rpm, the upper aqueous phase was removed with a Pasteur pipette, and the sedimented $CHCl_3$ phase was quantitatively transferred to a small vial with a microsyringe. The sample was blown dry with a gentle stream of nitrogen gas at room temperature. Derivatives of AFB_1 and AFB_2 were prepared by adding 200 μL *n*-hexane and 100 μL trifluoroacetic acid to the residue, heating the mixture at 40 °C in a water bath for 15 min, evaporating to dryness and then dissolving the residue with 200 μL of MeCN/MeOH/1% H_3PO_4 (17 + 13 + 70) and vortexing for 30 s. The solution was filtered with a 0.22 μm nylon membrane filter, transferred to the sample vial and analyzed by HPLC.

2.4. HPLC-FLD analysis

Aflatoxins (AFB_1 and AFB_2) were quantified according to the method described in our previous study (Lai et al., 2014) with minor modifications. HPLC analysis was performed with an Agilent 1260 HPLC system (Agilent Technologies, Germany) equipped with a quaternary pump, an automatic sample injector, a degasser, and a fluorescence detector. Separations were conducted with a KR100-10 C_{18} column (5 μm, 150 mm × 4.6 mm, Kromasil Limited). Acetonitrile (MeCN) was used as mobile phase A, methanol (MeOH) was used as mobile phase B, and 1% phosphoric acid (H_3PO_4) was used as mobile phase C. At first, the mobile phase was maintained for 13 min at a ratio of 17/13/70 (v/v/v) A/B/C, then changed to 45/15/40 (v/v/v) over a 2 min gradient and maintained for 1 min, then it was switched back to 17/13/70 (v/v/v) over a 2 min gradient and maintained for 5 min. The flow rate was set at 1.0 mL/min, and the injection volume was 50.0 μL. The detection wavelengths for AFB_1 and AFB_2 were 360 nm and 440 nm for excitation and emission, respectively. The retention time was 5.95 min and 13.98 min for AFB_1 and AFB_2, respectively (Fig. 1).

3. Results and discussion

Analysis of the aflatoxin-producing activity of *A. flavus* showed that 47 (37%) of 127 tested isolates produced aflatoxins, and the levels ranged from 175 to 124101 μg kg^{-1} for AFB_1 and 0 to 10329 μg kg^{-1} for AFB_2 (Table 1). Lisker et al. (1993) reported that *A. flavus* is often the most frequently isolated aflatoxin-producing species. Our results have supported the opinions, in which 37% of the tested *A. flavus* strains were found to produce aflatoxins on a rice substrate. Reddy et al. (2009c) also reported that 50.5% (43) of the tested *A. flavus* strains (85) isolated from discolored rice grains in

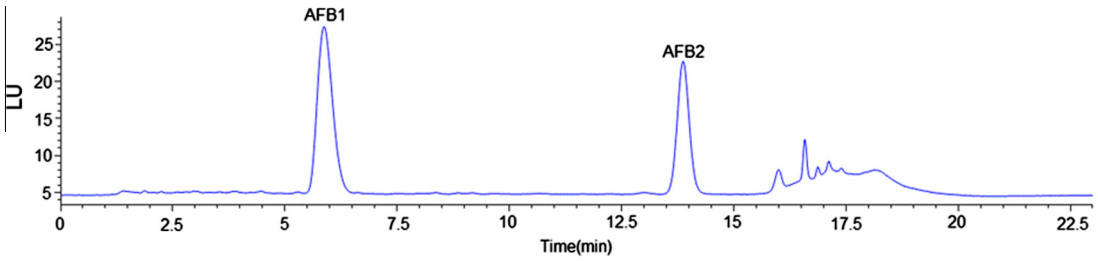

Figure 1 HPLC Chromatograms of AFB_1 and AFB_2 in polished rice grain culture. The retention times of AFB_1 and AFB_2 were 5.95 min and 13.98 min, respectively.

Table 1 Aflatoxin production (μg kg^{-1} dry rice grain) on polished rice grains by *A. flavus* strains isolated from rice collected from twelve provinces, China.

Source of places (province)	All tested isolates (No.)	Aflatoxigenic strains (No.)	Aflatoxins yields of aflatoxigenic strains			
			Maximum of AFB_1	Minimum of AFB_1	Maximum of AFB_2	Minimum of AFB_2
Guangdong	31	9	30797	1513	3856	ND[a]
Hunan	32	13	124101	175	10329	311
Jiangsu	11	2	5081	1409	5203	279
Yunnan	3	1	3257	3257	631	631
Sichuan	15	10	35263	473	9471	70
Jiangxi	2	1	2314	2314	1583	1581
Henan	4	0	/[b]	/	/	/
Guizhou	8	1	2020	2020	336	336
Anhui	3	2	21363	2372	8461	2030
Hubei	4	2	9292	2466	2109	866
Guangxi	6	3	11387	4799	6517	1450
Fujian	8	3	30156	1882	3079	1956
Total	127	47	124101	175	10329	ND
Average (mean)	47		10906 (5884)		2573 (1968)	

[a] ND, not detected.
[b] Means that there is no aflatoxigenic strains in Henan province.

India were identified as AFB_1 producers. Our results showed that the percentage of aflatoxigenic strains differs significantly in different provinces, for example, 29% in Guangdong, 40.6% in Hunan, 18.2% in Jiangsu, 66.7% in Sichuan, 12.5% in Guizhou, and 37.5% in Fujian province. However, none of the *A. flavus* strains in Henan province was an aflatoxigenic strain. We think that this was due to different brands and sources of the rice samples and the culture conditions. So, more strains need to be analyzed for their ability to produce aflatoxins.

In our study, the mean yields of the aflatoxin-producing isolates were 5884 μg kg^{-1}, 1968 μg kg^{-1} and 7852 μg kg^{-1} for AFB_1, AFB_2, and total AFBs (AFB_1 + AFB_2), respectively (Table 1). Our results are in accordance with other reports indicating that many strains of *A. flavus* isolated from corn and other widely varying substrates have a high potential for aflatoxin production (Joffe, 1969; Graciela et al., 2003; Reddy et al., 2009a,c, 2011). However, the production of aflatoxins by different strains of *A. flavus* varies widely. The *A. flavus* strains tested in our study produced AFB_1 in the range from 175 to 124101 μg kg^{-1} and AFB_2 from not detected to 10328 μg kg^{-1}. These results are in agreement with Abbas et al. (2005) who observed greater variations in aflatoxin production by *A. flavus*. Different aflatoxin production capabilities of the *A. flavus* strains would be influenced by different sources of the strains and environmental conditions.

The results of our study showed that 91.5% (43) of aflatoxigenic *A. flavus* strains produced higher levels of AFB_1 than AFB_2 in rice cultures. Only four isolates, which were isolated from Hunan, Sichuan and Fujian provinces, produced more AFB_2 (1251, 311, 514 and 1956 μg kg^{-1}) than AFB_1 (1166, 175, 474 and 1881 μg kg^{-1}) in rice culture. Among the tested *A. flavus* isolates, none of the four strains collected from the Henan province had the ability to produce aflatoxins. However, in the other eleven provinces, there has been at least one strain which can produce aflatoxins.

Rice and its products are primary foods for human consumption throughout the world, especially in China. Rice represents a very good substrate for fungal growth and toxinogenesis because it is used as an ideal culture growth medium to test the toxigenic potential of isolated strains (Bars and Bars, 1992). Our results showed that rice as a substrate is susceptible to AFB_1 and AFB_2 accumulation from *A. flavus* strains, and the mean yield of AFB_1 in rice grains from the *A. flavus* strains was 5884 μg kg^{-1}. One isolate of *A. flavus* (HN 65), which was isolated from Hunan province, produced the highest amount of AFB_1 (124101 μg kg^{-1}) and AFB_2 (10329 μg kg^{-1}) in rice cultures. In other studies,

Reddy et al. (2009c) reported that one isolate of *A. flavus* produced AFB_1 in the range 386000–415000 μg kg^{-1} on four rice cultures. Fouzia and Samajpati (2000) reported AFB_1 production by *A. flavus* on rice grains ranging from 555 to 10416 μg kg^{-1} from India. Reddy et al. (2011) observed AFB_1 accumulation from *A. flavus* on rice grains ranging from 3125.2 to 15645.2 μg kg^{-1}. All these results show that rice grain has a high risk of contamination by aflatoxins. In fact, many studies have reported that certain levels of aflatoxins have been detected in rice in many countries (Trucksee, 2000; Tanaka et al., 2007; Reddy et al., 2008).

4. Conclusion

The results of this study show that 37% (47) of the tested *A. flavus* strains produced aflatoxins, and most of the aflatoxigenic *A. flavus* strains showed a high capacity for aflatoxin production in rice grain. The high production of aflatoxins in rice grain cultures by *A. flavus* strains isolated from rice in China may have been due to incubation under optimal conditions. In fact, the levels of aflatoxins on rice samples in China are very low (unpublished data).

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