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Screening of aflatoxigenic fungi from Thai rice

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Abstract

Aflatoxins are extremely harmful and are the most carcinogenic substances known in nature, and as such, affect rice quality to a great extent. This study was undertaken to examine the presence of aflatoxigenic fungi in ten samples of long grain rice obtained from various provinces in Thailand. The rice grains were investigated directly for fungal colonization on agar plates. The major fungal genera detected were *Eurotium*, *Syncephalastrum,* and *Aspergillus*. Several isolates of *Aspergillus flavus*, a well-known aflatoxigenic species, were obtained from four rice samples. Aflatoxin production was confirmed in seven isolates using an HPLC fluorescence detection method. In all isolates, more than 1,000 ng/g of aflatoxin B_1 was detected, with 10-30 ng/g of aflatoxin B_2 also being detected, whereas aflatoxin G_1 and aflatoxin G_2 were under the limit of detection.

Key words: grain; mycoflora; aflatoxin; *Aspergillus flavus*; epiphyte

1. Introduction

Mycotoxins are a group of toxic fungal secondary metabolites, which can contaminate agricultural products under both pre- and postharvest conditions. They can cause acute, or chronic, carcinogenic, mutagenic, teratogenic, atherogenic, or oesteogenic toxic effects in both humans and animals (Hussein and Brasel, 2001; Kuiper-Goodman, 2004). Mycotoxins are produced by certain fungi (e.g., *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp.) that grow on human food, as well as on animal feed ingredients such as corn, sorghum, wheat, barley, peanuts, and other legumes and oilseeds. Five main groups of mycotoxins, namely aflatoxins, deoxynivalenol, ochratoxin A, fumonisins, and zearalenone, are commonly found in food and feed grains (Buzby 2003).

Aflatoxin is a potent human carcinogen produced by the genera *Aspergillus*, that causes both acute and chronic toxicity in humans and many other animals. At least 13 different types of aflatoxin are produced in nature, but only four are commonly found in agricultural commodities. These

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four main naturally-occurring aflatoxins are aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2. Among them, aflatoxin B_1 is the most common, as well as the one with the most potent ability to cause liver cancer in human. Aflatoxins are heat-resistant and can withstand exposure to normal cooking temperatures, as well as to microwave treatment (Midio, Campos, & Sabino, 2001). Aflatoxins M_1 and M_2 are metabolites of aflatoxin B_1 and B_2 in cattle animals, and are potentially important contaminants in milk, cheese, and other dairy products.

Many factors affect the growth of *Aspergillus* fungi and the level of aflatoxin contamination in food. Contamination can occur at any stage of the food production process from pre-harvest to storage (Wilson and Payne 1994). Aflatoxin contamination is also promoted by numerous events, including stress or damage to the crop due to drought prior to harvest, insect activity, incorrect time of harvest, heavy rains at harvest and post-harvest, and inadequate drying of the crop before storage (Hell, Cardwell et al. 2000; Ono, Sasaki et al. 2002; Hawkins, Windham et al. 2005; Turner, Sylla et al. 2005). Humidity, temperature, and aeration during drying and storage are also important factors that affect fungal growth and aflatoxin production. The viability of fungi is influenced by numerous factors, such as moisture level and pH; however, heating or cooking processes cannot be relied upon to destroy aflatoxins. For this reason, aflatoxins residue is a significant problem in processed foods.

Rice is one of the major cereals in the world, being a staple food throughout Asia, including Japan. Rice is of tropical origin and is generally tolerant to fungi in the field, compared with wheat and corn; however, mycotoxins are occasionally found (Tanaka et al. 2007). With respect to rice being affected by fungal species, the toxicity of moldy rice grains in Japan was first reported in 1891, (Sakaki 1891). The contamination of rice with aflatoxin is a serious concern in all countries that depend on rice as a staple in their diet. Contamination of commercial rice with aflatoxin has been reported throughout the world (Suarez-Bonnet et al. 2013, Lai et al. 2015). Among the four major aflatoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2), aflatoxin B_1 is classified as a group 1 (human) carcinogen by the International Agency for Research on Cancer (IARC) (IARC 1993).

Aflatoxin-contaminated rice is originally caused by adherence of aflatoxigenic fungi or epiphytes to rice grains. Because of the evidence of health risks, including carcinogenicity, the assessment of aflatoxin contamination in rice is essential. In this study, we screened for the presence of aflatoxigenic fungi in Thai rice and confirmed the presence of aflatoxin-producing isolates.

2. Materials and Methods

2.1. Microflora analysis

Thai rice (TR) grain samples (purchased at markets in Thailand in November 2016 and March 2017) were a kind gift from Prof. Yoshiko Sugita-Konishi, and ten samples were analyzed in this study. TR-1 was a sticky rice (Chiang Rai Province), TR-2 and TR-3 were Jasmine rice (Nakorusawan Province and Supanburi Province), TR-4 was a brown rice (Mae Teang City, Chiang Mai Province), TR-5 was a riceberry rice (Chiang Mai Province), TR-6 was a mixture of riceberry rice and hill tribe brown rice (Chiang Rai Province), TR-7 was a brown rice (Chiang Rai Province), TR-8 was a Jasmine rice (Bangkok), TR-9 was a mixture of lady ruby rice, jasmine brown rice, and fragrant rice (Chiang Rai Province), and TR-10 was a Jasmine rice (Chiang Mai Province). All rice grains were healthy in appearance and were kept at room temperature (below 26°C) until use. Fifteen grams of each rice grain were washed three times with distilled water. Five grains of Thai rice were placed directly into two kinds of culture media, Dichloran Rose Bengal Chroramphenicol (DRBC) agar or Dichloran Glycerol 18% (DG18) agar in quintuplicate, and screened for fungi after culture at 25°C for 7 days. After 7 days, colonized fungi were observed by microscopic examination and the fungal number was counted (Fig. 1) and was expressed as the colonization ratio% using the following equation.

Fungal colonization ratio (%) = (the number of grains with fungal colonization on the dish)/(total number of grains on the dish) x 100

Fungi with similar morphology were transferred to either Malt Extract Agar (MEA) or Czapek yeast extract agar (CYA) media for growth at 25°C for 7 days to allow for further identification of the fungal species from the genus *Aspergillus*. Possible aflatoxigenic fungi were then cultured on potato dextrose agar (PDA) to measure aflatoxin production *in vitro*.

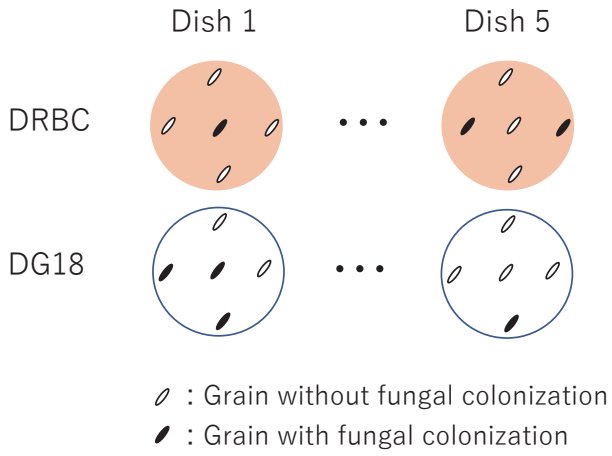

Fig. 1. Mycoflora analysis (5+5 dishes for each TR subsample)

2.2 Aflatoxin analysis of fungal isolates

Standard aflatoxin B_1 and aflatoxin G_1 were purchased from Acros Organics, (Geel, Belgium), whereas aflatoxin B_2 and aflatoxin G_2 were purchased from Wako, (Osaka, Japan). Aflatoxins were dissolved in acetonitrile as a working solution containing 50 ng/mL of each. All other reagents were of analytical or HPLC grade.

Possible aflatoxigenic fungal isolates from TR-7 (six isolates) and TR-10 (one isolate) were transferred to PDA, harvested after 5 days and killed by exposure to ammonia vapor. Approximately 2-5 g of scratched PDA, containing fungi, were placed into 50 mL centrifuge tubes. The samples were extracted with 5 mL of methanol, vortexed for 1 min, sonicated for 3 min, and centrifuged for 10 min. After centrifugation, 0.05 mL of supernatant was evaporated at 40°C under a gentle flow of nitrogen. Trifluoroacetic acid (TFA; 0.1 mL) was added to convert aflatoxin B_1/G_1 into the highly fluorescent hemiacetal aflatoxins B_2a/G_2a. The solution was then stored for 15 min at room temperature. A 0.9 mL aliquot of an acetonitrile and water solution (1:9, v:v) was added to the derivatized solution to allow for HPLC-fluorescence detection (HPLC-FL) (HPLC-FL system; Shimadzu, Kyoto, Japan).

HPLC-FL analysis was performed using an Inertsil ODS-3 column of 3.0 × 150 mm with a 4-µm particle size (GL Sciences, Tokyo, Japan). The composition of the mobile phase was acetonitrile, methanol, and water (10:30:60, v/v/v) with a flow rate of 0.3 mL/min. The column heater was set at 40°C, and the injection amount was 5 µL. The AF-TFA derivative solution was detected by FL absorption using wavelengths of 365 nm (excitation) and 450 nm (emission). Six standard concentrations of aflatoxin working solution, covering a range from 1 to 20 ng/mL (1, 2, 5, 10, 15, and 20 ng/mL), were employed, and calibration curves were plotted with peak areas and heights against the concentration of aflatoxins (ng/mL). The linearity of the calibration plot was demonstrated by calculating the correlation coefficient. The limit of detection (LOD) was defined as the concentration that was three times higher than the standard deviation of the blank signal. The LODs (ng/g) were 1.18, 0.55, 0.75, and 0.24 for aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2, respectively.

3. Results and Discussion

3.1 Mycoflora surveys

The data in Table 1 summarized the fungal colonization ratios obtained and show that they varied from 3.3% to 50%. Out of 500 rice grains tested, 335 grains showed fungal emergence. Among these 335 grains with colonized fungi, 167 grains were *Eurotium* spp., followed by 69 *Syncephalastrum* spp., 21 *Aspergillus* spp., and 11 *Penicillium* spp. The most prevalent fungi were *Eurotium* spp., being half of the epiphytes. The whole dataset showing the identified fungi is provided as Supplementary Table S1.

Table 1. Frequency of epiphytes on Thai rice grains

Fungi	Grain number	Ratio (%)
Eurotium spp.	167	50%
Syncephalastrum spp.	69	20.6%
Aspergillus spp.	21	6.3%
Zygomycetes spp.	20	6.1%
Wallemia spp.	18	5.4%
Cladosporium spp.	17	5.1%
Mucor spp.	12	3.6%
Penicillium spp.	11	3.3%
	335 (total)	

Among them, the genus *Aspergillus* was further observed to allow a detailed species level identification to be conducted. Table 2 showed the individual species of *Aspergilli* obtained from Thai rice grains.

Table 2. *Aspergilli* from Thai rice grains

Identified *Aspergillus* sp.	Grain number	Ratio (%)
Aspergillus flavus	8	38.1%
Aspergillus terreus	5	23.8%
Aspergillus oryzae	4	19.0%
Aspergillus ustus	1	4.8%
Aspergillus niger	1	4.8%
Aspergillus restrictus or *candidus*	1	4.8%
Aspergillus flavipes	1	4.8%
	21 (total)	

It was found that the most predominant *Aspergillus* sp. obtained from Thai rice grain subsamples was *Aspergillus flavus* (38.1%), followed by *A. terreus* (23.8%) and *A. oryzae* (19.0%). As shown in Table S1, we observed the adherence of *A. flavus* in four subsamples of Thai rice grains (TR-2, TR-4, TR-7, and TR-10). The highest frequency of *A. flavus* (a possible aflatoxin producer) as an epiphyte on grain was found in the subsample of TR-7 (on DRBC agar 5/25 (20%) and on DG18 agar 2/25 (8%)) (Table S1). As TR-7 is a white rice sample (Fig. 1), this demonstrates that polishing does not always lead to complete removal of fungal mycelia from the surface of grains, as reported in previous studies. In a study conducted in 2004, the incidence of fungal infection in Korean polished rice samples was examined. *P. citrinum* was the most frequently found species, with 27% of the 88 samples, followed by *A. candidus* (26%), *Alternaria* spp. (23%), and *A. versicolor* (20%) (Park et al (2005)). In a study by Ha et al. (1979), the frequency of the *Penicillium* genus increased as the storage moisture content increased from 14.5% to 26.9%. *Penicillium citrinum* and *A. candidus* were the predominant species infecting the samples, followed by *Alternaria* spp. and *A. versicolor*. In a study by Kushiro et al. (2015), *P. citrinum* (a possible citrinin producer) and *P. islandicum* (a possible luteoskyrin, cyclochlorotine producer) were isolated from Thai rice grains, but they were not found in this study.

In freshly harvested rice, *Fusarium* fungi are dominant as the mycotoxigenic fungi, whereas *Aspergillus* or *Penicillium* fungi are frequently found in rice during transportation and storage (Directorate-General Health and Consumer Protection, EU 2003). In accordance with the fact that these grains had been stored for over one year, we did not find any *Fusarium* fungi (Table S1). In this study, the isolation ratio of the *Penicillium* genus was very low (3.3%) (Table 1). This may arise from the storage conditions used (temperature, moisture, ventilation, and so on). The most prevalent epiphytic fungus in this study was *Eurotium* sp., which is the sexual state of *Aspergillus*. Since *Eurotium* species are xerophilic, they can co-occur with *Aspergilli* and survive over a year on rice grains under conditions of low moisture.

3.2 Aflatoxin analysis of fungal isolates

Among grains in which the adherence of *A. flavus* was found, six isolates from TR-7, and one isolate from TR-10, were investigated for aflatoxin production *in vitro*. Isolates were inoculated on agar plates and harvested after 5 days. A methanol extract of the scratched agar debris with fungal fragments was analyzed by HPLC-FL after TFA derivatization (Fig. 2). As shown in Fig. 2 and Table 3, a significant amount (>1,000 ppb) of aflatoxin B_1 was detected in all isolates, followed by a small amount (10-30 ppb)

Table 3. Aflatoxin production of the tested isolates

		Concentration of analyte (ng/g) (Area)				
Pre-culture	Isolate	Aflatoxin G_1	Aflatoxin B_1	Aflatoxin G_2	Aflatoxin B_2	Scratched agar (g)
PDA	7-5-4	<LOD	1328.3	<LOD	9.3	4.52
CYA	7-5-5	<LOD	1649.1	<LOD	12.1	3.20
DRBC	7-5-5	<LOD	1856.9	<LOD	12.7	3.10
PDA	7-4-5	<LOD	1972.4	<LOD	11.6	3.16
CYA	7-4-5	<LOD	3968.3	<LOD	29.6	2.81
MEA	7-5-5	<LOD	2459.5	<LOD	16.0	2.64
CYA	10-1-3	<LOD	2574.5	<LOD	18.8	4.14

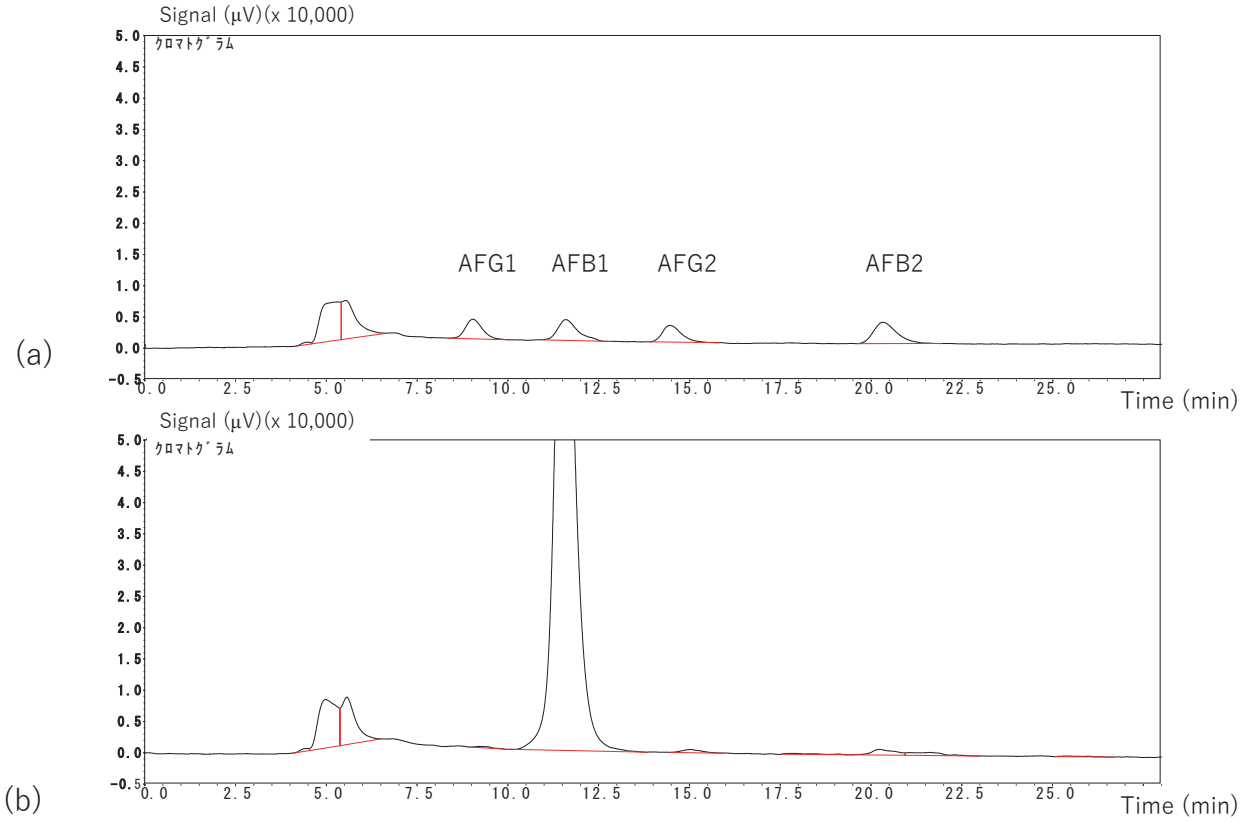

Fig. 2. HPLC-FL chromatograms of (a) standard solutions of aflatoxins (2 μg/mL each) and (b) TR-7-5-4 extract

of aflatoxin B_2. The production of G-type aflatoxins was under the LOD, supporting the fact that these isolates are *A. flavus* which lacks the biosynthetic genes necessary for the production of G-type aflatoxins.

As a result, seven of the *A. flavus* isolates investigated proved to be aflatoxigenic. Although all TR grain samples were stored at room temperature for over a year and appeared healthy, *A. flavus* survival and aflatoxin production *in vitro* were observed. These fact indicate that there is a high survival rate of the toxigenic *A. flavus* during storage of TR grains. These analyses of survival rate and aflatoxin production of aflatoxigenic fungi under various storage conditions in grains should be clarified in further studies.

4. Conclusions

In this study, we investigated the presence of epiphytic fungi on Thai rice and found that *Aspergillus* spp., with putative aflatoxigenic isolates, were the third most predominant epiphytes on grains followed by *Eurotium* spp. and *Syncephalastrum* spp. Among the *Aspergillus* sp., *A. flavus* was the most frequently found species. Our investigation demonstrates that Thai rice could therefore be a source of exposure to aflatoxin. Aflatoxigenic *A. flavus* was viable during long-term storage of Thai rice at room temperature, so it may be necessary to clarify the factors that affect their viability and aflatoxin production.

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Supplementary Table S1. Fungal isolation and identification

Sample ID	Media	Incidence (%)	Fungi detected																	
TR-1	DRBC	22/25 (88)	*Syncephalastrum racemosus*	8/25(32)	*Eurotium spp.*	1/25(4)	*Aspergillus flavipes*	1/25(4)	*Penicillium implicatum*	1/25(4)	Unknown	8/25(32)								
	DG18	25/25 (100)		3/25(12)		17/25(68)		0/25 (0)		0/25 (0)		3/25 (12)								
TR-2	DRBC	3/25 (12)	*Wallemia spp.*	9/25(36)	*Eurotium spp.*	2/25(8)														
	DG18	7/25 (28)		9/25(36)		2/25(8)														
TR-2	DRBC	8/25 (32)	*Eurotium chevalieri*	1/25(4)	*Penicillium aurantiogrisum*	1/25(4)	*Aspergillus terreus*	1/25(4)	*Syncephalastrum spp.*	0/25(10)	*Cladosporium spp*	0/25 (0)								
	DG18	8/25 (32)		1/25(4)	*Penicillium thomii*	1/25(4)		1/25(4)		1/25(4)		1/25 (4)								
					Penicillium brevicompactum	1/25(4)														
TR-2	MEA				*Penicillium griseofuivum*	1/25(4)	*Aspergillus flavus*	1/25(4)												
TR-3	DRBC	1/25 (4)	*Cladosporium sp.*	1/25 (4)																
	DG18	0/25 (0)		0/25 (0)																
TR-4	DRBC	25/25 (100)	*Eurotium spp.*	0/25(0)	*Aspergillus flavus*	0/25(0)	*unknown*	25/25(100)												
	DG18	25/25 (100)		25/25(100)		0/25 (0)		0/25 (0)												
	MEA					1/25(4)														
TR-5	DRBC	25/25 (100)	*Eurotium spp.*	14/25(56)	*Syncephalastrum racemosus*	12/25(48)	*Aspergillus oryzae*	2/25(8)	*Aspergillus niger*	1/25(4)	*Zygomycetes or Rhizopus spp.*	15/25(60)	*Mucor sp.*	0/25(0)						
	DG18	25/25 (100)		2/25(8)		25/25(100)		0/25 (0)		0/25 (0)		0/25 (0)		1/25 (4)						
TR-6	DRBC	18/25 (72)	*Eurotium spp.*	4/25(16)	*Penicillium glabrum*	1/25(4)	*Aspergillus terreus*	3/25(12)	*Syncephalastrum spp.*	4/25(16)	*Mucor spp.*	6/25(24)	*Rhizopus sp.*	1/25(4)	Unknown	0/25(0)				
	DG18	25/25 (100)		11/25(44)		0/25(0)		0/25 (0)		4/25(16)		4/25 (16)		0/25 (0)		6/25 (24)				
TR-7	DRBC	11/25 (44)	*Eurotium spp.*	2/25(8)	*Penicillium glabrum*	1/25(4)	*Aspergillus flavus*	5/25(20)	*Syncephalastrum racemosus*	0/25(0)	*Cladosporium cladosporoides*	0/25 (0)	*Mucor racemosus*	0/25(0)	*Aspergillus oryzae*	0/25(0)	Unknown	3/25(12)		
	DG18	12/25 (48)		4/25(16)		2/25(8)		2/25 (8)		1/25(4)		1/25 (4)		1/25 (4)		1/25 (4)		1/25 (4)		
TR-8	DRBC	0/25 (0)	*Eurotium sp.*	0/25(0)	*Penicillium griseofuivum*	0/25(0)	*Aspergillus restrictus or Aspergillus candidus*	0/25(0)	*Cladosporium spp.*	0/25 (0)										
	DG18	11/25 (44)		1/25(4)		1/25(4)		1/25 (4)		7/25 (28)										
TR-8	MEA							1/25(4)												
TR-9	DRBC	19/25 (76)	*Eurotium spp.*	11/25(44)	*Aspergillus oryzae*	0/25(0)	Unknown	3/25(12)	*Cladosporium spp.*	0/25 (0)	*Zygomycetes or Rhizopus spp.*	4/25(16)								
	DG18	25/25 (100)		16/25(64)		1/25 (4)		0/25 (0)		8/25 (32)		0/25 (0)								
TR-10	DRBC	3/25 (12)	*Penicillium implicatum*	0/25(0)	*Aspergillus flavus*	1/25(4)	*Aspergillus ustus*	1/25(4)												
	DG18	0/25 (0)		1/25(4)		0/25 (0)		0/25 (0)												

和文要旨

アフラトキシンは有害かつ最も発がん性の高い天然物で、コメの甚大な品質低下を招きうる。タイの各地方から入手した長粒種の穀粒サンプル 10 点で、アフラトキシン産生菌の調査を行った。コメ穀粒を直接、寒天平板培地に置いて調査した。主な検出菌は、*Eurotium* 属、*Syncephalastrum* 属、*Aspergillus* 属であった。アフラトキシン産生で知られる *Aspergillus flavus* が、4 点の試料より数株得られた。HPLC- 蛍光検出法によって、7 菌株のアフラトキシン産生能を確認した。全ての菌株で 1,000 ng/g 以上のアフラトキシン B_1 と 10-30 ng/g のアフラトキシン B_2 が検出された。いっぽうアフラトキシン G_1 とアフラトキシン G_2 は、検出限界未満であった。