Note

Microflora of Mycotoxigenic Fungi in Rice Grains in Kyushu Region of Japan and Their Changes during Storage under non-Controlled Conditions

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Contamination of agricultural crops by mycotoxins has increased because of the expansion of mycotoxin-producing fungi along with global warming. In this study, the fungal microflora of brown rice grains cultivated in Kyushu region in the southern part of Japan was investigated. A total of 75% of rice samples examined in this study showed less than 30% of fungal contamination rates with a median rate of 12.5%. Some isolates of *Aspergillus flavus* showed the ability to produce aflatoxins (AFs) (AFB1 production was 62.5-70.4 ng/mL). Furthermore, AF-producing *Aspergillus flavus* survived during storage and *Aspergillus creber*, which produced sterigmatocystin, was detected in a stored rice sample. Although AFs or sterigmatocystin-contamination was not detected in any rice samples, these mycotoxin-producing fungi are distributed and can survive during storage under the natural conditions in Japan. Employing suitable storage conditions is important for preventing mycotoxin contamination of brown rice grains.

Key words : Kyushu region of Japan / Microflora change / Rice / Storage.

Rice is a staple food for more than half of total world population, including Japan, and is one of the most consumed cereals worldwide. In terms of cultivation and consumption, approximately 700 million tons of rice are produced globally (FAO, 2018). Although mycotoxin contamination in rice occurs at less than half the rate observed in wheat and corn, many studies reported the contamination of rice by mycotoxins such as aflatoxins (AFs), citrinin, ochratoxin A, fumonisins, and

trichothecens, as well as by mycotoxigenic fungi (Tanaka et al., 2007; Reddy et al., 2008). Among the mycotoxins contaminating rice, the most threatening are AFs, which are highly toxic and carcinogenic. One type of AFs, AFB1, is classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002). However, AFs have not threatened Japanese domestic rice grains because AF-producing fungi such as *Aspergillus flavus* are distributed mainly in tropical and subtropical regions. AF-contamination of food and feed are rare in Japan. However, because of global warming, there are increased concerns that AF-producing fungi may spread to

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TABLE 1. Contamination rates of rice samples (before storage)

No.	Contamination Rate (%)							Aw
	<i>Asp</i>	<i>Pen</i>	<i>Cla</i>	<i>Eur</i>	<i>Fus</i>	Other	Total	
7	-	-	-	-	-	1	1	0.66
8	1	-	-	-	-	-	1	0.65
9	-	1	-	-	-	2	3	0.68
1	2	-	2	-	-	-	4	0.70
5	4	-	-	-	-	-	4	0.67
6	-	-	-	-	-	4	4	0.66
11	1	2	-	-	-	1	4	0.76
10	-	4	-	-	-	1	5	0.62
2	5	1	-	-	-	-	6	0.67
4	-	-	4	-	-	2	6	0.78
25	-	5	-	-	-	2	7	0.60
27	-	-	-	-	-	7	7	0.66
28	1	4	-	-	-	2	7	0.62
12	-	-	4	4	-	4	12	0.70
14	8	-	-	3	-	2	13	0.68
24	-	-	4	6	1	2	13	0.74
26	-	-	-	10	-	4	14	0.61
15	4	4	1	-	-	10	19	0.77
16	-	-	-	-	-	26	26	0.66
19	2	-	-	4	-	22	28	0.74
21	4	-	2	-	-	22	28	0.77
13	6	10	-	-	-	13	29	0.69
3	10	2	-	-	1	21	34	0.62
18	-	-	-	6	-	38	44	0.66
23	-	6	-	2	1	47	56	0.74
17	8	-	-	-	-	60	68	0.90
20	4	52	-	-	-	18	74	0.69
22	14	-	-	-	-	78	92	0.69

* *Asp*: *Aspergillus*, *Pen*: *Penicillium*, *Cla*: *Cladosporium*, *Eur*: *Eurotium*, *Fus*: *Fusarium*

temperate regions. Indeed, the natural occurrence of AF contamination in rice grains was reported in Asian countries such as Korea and China (Oh et al., 2010; Lai et al., 2015; Kim et al., 2017; Sun et al., 2017). In Japan, AFB1 was also found in rice harvested from a field at the University of Miyazaki, which is the southern part of Japan, in 2011. These data suggest the expanding distribution of AF-producing fungi, but few studies have examined the microflora of recent rice grains or fields in temperate regions. In this study, we analyzed the fungal microflora of rice grains cultivated in Kyushu region, in the southern part of Japan, where concerns have been raised regarding the effects of global warming.

Twenty-eight rice commodities cultivated in Kyushu

region in 2013 and 2014-year crop were used (Table 1). The fungal microflora in brown rice grains were analyzed as follows. One hundred rice grains in each sample were exposed to 70% ethanol solution for 30 s and washed twice with sterile distilled water. Five rice grains per one plate were placed onto dichloran rose bengal chloramphenicol agar or dichloran 18% glycerol agar medium and cultured at 25°C for 5 days. After incubation, developed fungi were counted and to analyze the mycoflora, the developed fungi were identified at the genus level as *Aspergillus*, *Penicillium*, *Cladosporium*, *Eurotium*, *Fusarium*, and others by microscopic observation.

Furthermore, changes in the fungal microflora during

storage were analyzed. In Japan, rice is generally stored in an air-conditioned environment at a temperature of approximately 15°C and relative humidity (RH) of approximately 70%. Thus, fungal contamination of rice grains rarely increases during storage in Japan. However, storage under natural condition is common among small farmers. In these cases, fungal contamination may increase, but few studies have examined the changes in the fungal microflora of rice grains during storage under natural conditions. The rice grains used in this study were placed in paper bags and stored in a barn from November 2014 to October 2015 (for 10 months). Changes in the fungal flora of rice grains during storage without control of temperature and humidity in the barn located in Kanagawa Prefecture, eastern part of Japan, was investigated.

Before storage, the contamination rate of approximately half of the samples (13/28 rice samples) was less than 10%, although the fungi developed on 92% of rice grains in samples showing the greatest contamination (Table 1). Additionally, 75% of samples showed fungal contamination rates of less than 30% with a median of 12.5% (Fig. 1A). At the genus level, *Aspergillus* species were most frequently detected (15/28), followed by *Penicillium* (11/28), *Eurotium* (7/28), *Cladosporium* (6/28), and *Fusarium* (3/28) (Table 2). To examine contamination by AF-producers such as *A. flavus*, *Aspergillus* isolates were identified at the species level. The isolates were transferred to potato dextrose agar, Czapek yeast agar, and malt extract agar media and cultured at 25°C for 7 days. Colonies were identified based on morphological characteristics. *Aspergillus flavus* was detected in four rice samples, indicating that these rice samples were possible to be contaminated by AFs. Previously, most *A. flavus* isolated from foods and soils in Japan were thought to be non-AF-producing strains (Manabe et al., 1976; Saito et al., 2008). However, recent studies showed that AF-producing *A. flavus* was detected near Okinawa prefecture and the Kyushu region, in the south of Japan, and sometime in the northern part of Japan (Okazaki et al., 1992; Takahashi, 1993; Takahashi et al., 2004). The isolates identified as *A. flavus* in this study may produce AFs. AF production by these isolates was investigated by high-performance liquid chromatography as described by Yabe et al. (1988). All isolates produced AFB1 in the range of 62.5–70.4 ng/mL (average 67.5 ± 3.0 ng/mL). AFs were not detected in rice samples contaminated by these *A. flavus* isolates. This may be because of the low contamination rates of $2.8 \pm 1.5\%$ (Table 2).

After 7 months of storage (until June 2015), the fungal contamination rates were decreased in more than half of the rice samples (15/28), and the median contami-

FIG. 1. Fungal contamination rate, conditions in the barn, and water activity of rice grains.

A: Transition of fungal contamination rate of rice grains. B: Transition of temperature and relative humidity in the barn. C: Transition of water activity of rice grains. In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the maximum and minimum.

nation rate decreased to 8.5% (Fig. 1A). The storage period from November to June is autumn to spring in Japan, which are relatively dry and cold seasons. RH in the barn was consistently lower than 70% although it was relatively higher in February possibly because of snow, and the temperature was lower than 15°C before

TABLE 2. Changes of contamination level during storage

Genera (species)	No. of contaminated samples (Contamination rate) ^a		
	Before storage	7 months	10 months
<i>Aspergillus</i>	15 (4.9 ± 3.7%)	19 (1.7 ± 3.6%)	15 (4.5 ± 6.1%)
(<i>Aspergillus flavus</i>)	4 (2.8 ± 1.5%)	9 (1.3 ± 0.5%)	0 -
(<i>Aspergillus versicolor</i>)	0 -	1 (1.0%)	1 (3.0%)
<i>Penicillium</i>	11 (8.3 ± 14.7%)	8 (2.4 ± 2.1%)	3 (1.3 ± 0.6%)
<i>Cladosporium</i>	6 (2.8 ± 1.3%)	1 (1.0%)	0 -
<i>Fusarium</i>	3 (1.0 ± 0.0%)	4 (1.3 ± 0.5%)	1 (1.0%)
<i>Eurotium</i>	7 (5 ± 2.6%)	14 (2.2 ± 2.8%)	26 (39.2 ± 16.8%)
Other	24 (16.3 ± 20.7%)	25 (15.4 ± 19.2%)	22 (16.1 ± 16.4%)

^a average ± SD

May (Fig. 1B). The contaminating fungi abundance decreased during storage even under natural conditions possibly because of the low temperature and RH. After 10 months of storage, the contamination rates had increased in most rice samples (26/28), and the median of fungal contamination rate increased to 50% (Fig. 1A). This may be because of the rising temperatures and degree of humidity in the barn. In Japan, June is the rainy season, after which summer begins. As shown in Fig. 1B, the temperature in the barn began increasing to greater than 25°C, which is an appropriate temperature for fungal growth. The humidity also began increasing and reached more than 70% after June, and the water activity (*Aw*) of rice grains increased with rising temperature and humidity (Fig. 2C). Although the median *Aw* of rice grains before storage was 0.68, this value increased to 0.82 after 7 months of storage and was maintained at a high level (0.75) until 10 months of storage.

Examination of contamination by specific genera revealed that the detection rates of *Aspergillus* (19/28) and *Eurotium* (14/28) increased and those of *Penicillium* (8/28) and *Cladosporium* (1/28) decreased after 7 months of storage. After 10 months of storage, the contamination rates of all examined genera except for *Eurotium* decreased. During storage, the abundance of fungi contaminating in the field was thought to decrease; particularly, the detection of *Cladosporium*,

which is one of the major fungi present in the field, decreased from that in six rice samples to that in only one rice sample after 7 months of storage, and not detected after 10 months of storage. Other filed fungus *Fusarium* was detected in only one sample after 10 months of storage. On the other hand, *Eurotium* was detected in nearly all samples (26/28) and the contamination rate increased from 2.2±2.8% to 39.2±16.8% after 10 months of storage. Xerophilic fungus such as *Eurotium* increased in the fungal flora of rice grains as *Aw* decreased during storage (Table 2, Fig. 1C).

After 7 months of storage, nine rice samples were contaminated with *A. flavus*. Although *A. flavus* was newly detected in seven rice samples in which *A. flavus* was not detected before storage, two rice samples have been contaminated with *A. flavus* before storage, indicating that *A. flavus* can survive during storage in temperate regions. Furthermore, the levels of *A. flavus* in newly contaminated seven samples may have been below the detection limit before storage, and the fungi survived and their abundance increased during storage. These isolates produced AFB1 in the range of 55.8–70.2 ng/mL. Sugihara et al (2016) stored rice artificially contaminated with AF-producing *A. flavus* under natural conditions in Japan and found that AF-contamination in rice can occur in temperate regions. Our data suggest that *A. flavus* can survive during storage even in natural occurrence with a low contamination rate. Although AFs

were not detected in these contaminated rice samples after storage, when the contamination rate in the field increases and storage conditions are more suitable for fungal growth, the rice grains can become contaminated by AFs during storage, even in temperate regions. The optimum temperature for the growth of *A. flavus* was 30-35°C (Abdel-Hadi et al., 2012; Ayerst 1969) and the optimum temperature for AFB1 production on rice was reported to be 28°C (Sorenson et al., 1967).

Additionally, *A. versicolor*, which is known as a sterigmatocystin (STC)-producer (Rank et al., 2011), was isolated from one rice sample stored for 7 months and one sample stored for 10 months. STC is known as a precursor to AFB1 and is carcinogenic, categorized as a 2B carcinogen (IARC, 1987). Some studies reported the natural occurrence of STC contamination by *A. versicolor* in rice grains subjected to long-term storage in some Japanese storehouses at room temperature (Manabe et al., 1976; Sugimoto et al., 1977). Similarly, this species was detected only in stored samples in this study. This species may survive and increase in number during long-term storage. STC-production of the isolates morphologically identified as *A. versicolor* in this study was also investigated. The results showed that one of four isolates produced STC at a concentration of 330 ng/mL. Recently, the section including *A. versicolor*, *Versicolores*, was taxonomically revised based on molecular phylogenetic analysis, and the STC production of each species was investigated (Jurjevic et al., 2012; Jurjević et al., 2013). The *A. versicolor* isolates in this study were identified based on a molecular phylogenetic method using the β -tubulin gene as previously described (Kobayashi et al., 2018). As a result, isolates producing STC were identified as *A. creber*, which was described as an STC-producer in a previous study (Jurjević et al., 2013). Another isolate that did not produce STC was identified as *A. sydowii*, which has been recognized as a non-STC producer (Frisvad and Thrane, 1995). STC was not detected in rice grains contaminated with *A. sydowii* or *A. creber*. Although no STC contamination was detected because of the low contamination rate by *A. creber* (1%), *A. creber* levels may increase and cause STC contamination under unsuitable storage conditions. The optimum growth temperature for *A. versicolor* are 30°C and the optimum temperature for STC production by *A. versicolor* was 27 to 29°C (Rabie et al., 1976; Versilovskis and De Saeger, 2010).

Additionally, some species in the genus *Penicillium* produce mycotoxins, particularly the infamous case of molded toxic yellowed rice in Japan. To date, three historical cases have been documented in imported rice grains, and three causative species were identified: *Penicillium citreonigrum* producing citreoviridin,

Penicillium islandicum producing luteoskylin and cyclochlorotine, and *Penicillium citrinum* producing citrinin (Udagawa and Tatsuno, 2004; Kushiro, 2015). Next, we examined whether these three species could be detected among the isolates morphologically identified as *Penicillium*. *Penicillium* species causing toxic yellowed rice have never been detected in any rice samples regardless of whether they were stored. However, these *Penicillium* species required extra attention because they are possible to be expanded to Japan according to the effects of global warming.

In this study, the fungal microflora of rice grains cultivated in the southern part of Japan was investigated. We found that AF-producing *A. flavus* and STC-producing *A. creber* were distributed in this region, indicating the potential for contamination of Japanese domestic rice by AFs or STC. However, no AF or STC contamination was observed in rice grains investigated in this study because of the low contamination rate. Furthermore, these fungi can survive during storage under natural conditions in Japan. Mycotoxin contamination can occur when storage conditions are unsuitable. Storage under controlled conditions is important even in temperate regions to prevent mycotoxin contamination.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ABBREVIATIONS: AF: aflatoxin, STC: sterigmatocystin

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