Studies on the Population of Toxigenic Fungi in Foodstuffs III. Mycoflora of Milled Rice Harvested in 1965

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The mycoflora of 219 samples of milled rice harvested in 1965 was determined. Samples in domestic channels were collected from each of the nine geographical areas of Japan. To determine the fungal flora, fifty grains of rice from each sample were plated on Peptone-glucose agar containing chloramphenicol. Fungi were found in 143 of the samples (66.3%), whereas 76 (34.7%) did not yield any fungi. Of the 143 positive samples, 119 samples (83.2%) were usually associated with one to five colonies per 50 grains. No particular relation existed between the frequency of occurrence of fungi and the geographical areas. The total number of fungal isolates was 835 involving more than 25 genera. The major genera of fungi among these isolates were *Aspergillus* (51.0%), *Catenularia* (11.0%), *Penicillium* (9.2%), *Fusarium* (4.9%) and *Cladosporium* (4.8%). Other genera such as *Helminthosporium* (4.0%), *Trichoconis* (2.4%), *Phoma* (2.4%), and *Piricularia* (1.1%) were frequently isolated. The comparatively low incidence of fungi suggests that most of the samples had been kept under suitable storage conditions after harvesting and handling. *Aspergillus repens* (67.1%) was the most prevalent among 426 isolates of *Aspergillus*, and *Penicillium canescens* series (27.3%) was the major group of *Penicillium* spp. but *Penicillium citrinum* (3.2%) and *Penicillium cyclopium* (2.3%) were more widely distributed in the samples than *Penicillium canescens*. On the basis of these results, the milled rice investigated in this study was considered to have been conditioned and handled under satisfactory conditions.

Introduction

Since the discovery of fungal metabolities that are hepatotoxic and carcinogenic[1)~8)], the importance of fungi inhabiting various kinds of foodstuffs has been increasing in the field of the food hygiene. The problem of mycotoxicoses has now a world-wide significance in terms of public health, agriculture, economic, etc. Because rice is one of the major crops in Japan, it was thought that studies of the mycoflora of rice grains would be of great value, particularly as they are related to the fungal toxin production.

Rice grains infested with seedborne fungi have been studied by many plant pathologists and some of strains of pathogenic fungi belonging to the genera of *Helminthosporium*, *Curvularia*, *Alternaria*, and *Fusarium* were associated with various types of discolored rice seeds[9)10)]. Quite recently Schroeder[11)] reported the orange stain seed caused by *Penicillium puberulum*. In relation to the fungal deteriolation of stored rice, extensive studies have been carried out by many investigators such as Del Prado and Christensen[12)] and Kurata et al.[13)] in the United States. Tsunoda et al.[14)] studied imported rice from over seventeen foreign countries and Tsuruta[15)~17)] investigated domestic brown-rice and imported rice in 1956~1958 in Japan. Results of these investigations indicated that species of *Aspergillus* and *Penicillium* made up the bulk of the isolations in all of the samples examined but *Fusarium*, *Cladosporium*, *Curvularia* and *Streptomyces* were also found in significant numbers. Taxonomical studies of fungi from stored rice were made by Hirayama and Udagawa[18)19)]. Udagawa[20)]

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described five new species of *Penicillium* isolated from rice collected in 1955~1957, in addition to many isolations of previously known species. In the field of food hygiene, mycological examinations have been done by Iizuka[21)22)] in Thailand and Burma rice and by Inagaki[23)] in imported rice in general. Their results showed that toxigenic fungi such as *Penicillium islandicum*[2)4)5)], *Penicillium citrinum*[3)], *Penicillium toxicarium* (*P. citreo-viride*)[3)], and *Aspergillus flavus*[24)25)7)] were commonly isolated from the samples, but they did not investigate the toxigenicity of their isolates in animal tests.

The final object of this study is to determine the prevalence of toxic molds in Japanese milled rice available for daily utilization in the market. Acute toxicity tests of the representative strains of fungi isolated from rice is now being made on laboratory animals. Only the results of the mycological examination of milled rice are presented here.

Materials and Methods

Sampling. A total of 219 bags of milled rice from 132 Prefectural Public Health Stations located in nine geographical regions of Japan (Fig. 1) were sampled. For our examinations of rice mycoflora, sampling from the various geographical regions was taken into consideration, but rice varieties and product origins were not considered. All of the samples were collected in the fall of 1965 and have been stored in the laboratory from December of 1965 to August of 1966.

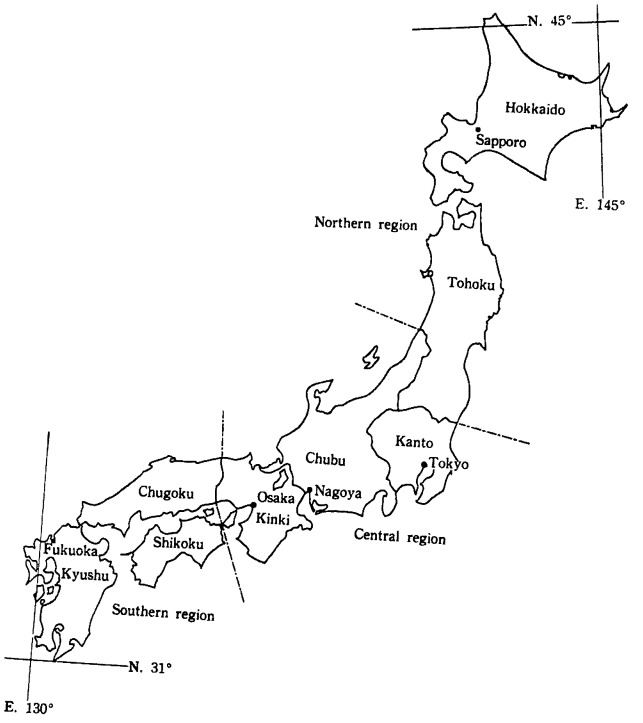

Fig. 1. Map of sampling locations

Procedure for rice fungi examination. The rice was plated following surface washing with sterile water as has been established by Kurata et al.[13)] and Tanaka et al.[26)] The samples were cultured on Peptone-glucose agar containing 100 mg per liter of chloramphenicol to suppress bacterial growth. Prior to culturing, about 10 g of rice grains were washed asceptically with ten successive volumes of sterile distilled water to removed surface contaminates. Five grains were placed into each of 10 Petri dishes. These dishes were incubated at 25°C, and inspected daily. Further inspection of the dishes were made after 10 days and the more slowly developing colonies were transferred to PDA (Potato-Dextrose agar) slants and cultured for identification. If necessary they were again transferred to adequate media for final identification. All isolates were maintained on PDA medium under low temperature until determination of their toxigenicity was accomplished. *Aspergillus* and *Penicillium* isolates were identified by consulting descriptions[27)28)] for diagnosis and the use of the com-

monly employed spot and slide culture method using the following agar media: Czapek's solution, malt extract, potato dextrose and corn meal.

Moisture contents. These were determined electrically by the "Kett" cereals moisture meter at the beginning of all tests.

Results and Discussion

Of the 219 samples of rice, fungi were isolated from 143 samples, whereas 76 samples did not yield fungi. Thus, fungi were not isolated from 34.7 percent of the total samples. Relation between the total number of fungi isolated and the sampling areas is shown in Table 1. The sampling areas are arranged in order from the southern region to the northern region of Japan. It was noted that there were no significant differences between the frequency of the occurrence of fungi and the sampling areas, except Hokkaido in the northern region. Samples were collected in Hokkaido in the beginning of August and moisture content of the samples was rather high, about 16.0%. As a result, the occurrence of fungi was high in some of the sam-

Table 1. Relation between the Total Number of Fungi and Sampling Area of Milled Rice Collected in 1965

Sampling areas	No. of samples examined	No. of fungus colonies isolated (50 grains)								Range of moisture content(%)
		0	1~5	6~10	11~15	16~20	21~30	31~50	51~	
		%	%	%	%	%	%	%	%	
Southern region										
Kyushu	16	25.0	68.8	6.3						12.8~13.9
Chugoku	22	13.6	72.7	13.6						12.9~14.2
Shikoku	13	38.5	46.2	15.4						13.2~13.9
Central region										
Kinki	25	24.0	64.0	4.0	4.0		4.0			12.9~14.1
Chubu	32	28.1	68.8	3.1						12.7~14.8
Kanto	20	25.0	70.0				5.0			12.6~14.2
Tokyo	45	57.8	40.0		2.2					14.0~15.2
Northern region										
Tohoku	20	40.0	60.0							13.3~14.7
Hokkaido	26	38.5	15.4		11.5	7.7		19.2	7.7	13.3~16.3
Total	219	34.7	54.3	3.7	2.3	0.9	0.9	2.3	0.9	12.6~16.3

Table 2. List of the Genera and the Frequency of Occurrence of Fungi Isolated from Rice Grains

Fungi isolated	No. of isolates	% of occurrence	Fungi isolated	No. of isolates	% of occurrence
Aspergillus	426	51.0	*Mucor*	3	0.4
Catenularia	92	11.0	*Epicoccum*	2	0.2
Penicillium	77	9.2	*Nigrospora*	2	0.2
Fusarium	41	4.9	*Scopulariopsis*	1	0.1
Cladosporium	40	4.8	*Doratomyces*	1	0.1
Helminthosporium	33	4.0	*Gliomastix*	1	0.1
Trichoconis	20	2.4	*Chrysosporium*	1	0.1
Phoma	20	2.4	*Septoria*	1	0.1
Piricularia	9	1.1	*Rhizopus*	1	0.1
Curvularia	7	0.8			
Alternaria	7	0.8			
Chaetomium	7	0.8	Mycelia sterilia	19	2.3
Coniothyrium	3	0.4	Unidentified	21	2.5
			Total	835	

ples from Hokkaido. In a previous study by Del Prado and Christensen[12], the critical moisture for the growth of molds on rice is somewhere between 14 and 16%. It was noteworthy that one to five colonies were isolated from 54.3% of samples examined. This average occurrence of fungi (1~5 colonies per samples) was in 83.2% of the total positive samples; indicating that most of the samples examined had been kept under suitable conditions after harvesting and handling.

The genera and distribution of the fungi isolated are listed in Table 2. From the total of 835 isolates recorded during the examination, it was possible to identify twenty-two genera of fungi. It will be noted that *Aspergillus* (51.0%), *Penicillium* (9.2%), *Fusarium* (4.9%), and *Cladosporium* (4.8%) were the predominate genera. Among these genera, *Aspergillus* spp. were most frequent fungi occurring 23.3% per the samples examined. Similar results have been reported by other workers from other countries[12)~23)]. *Helminthosporium* spp. were found in 4.8%. The other pathogenic groups of fungi such as *Trichoconis*, *Piricularia* and *Curvularia* were moderately prevalent. *Chaetmium* (0.8%) appeared in small numbers. The remaining genera of fungi occurred occasionally accounting for less than 1.0% of the total isolations.

Table 3 presents a list of the species of *Aspergillus* found and indicates their frequency of occurrence in rice grains. A total of 426 colonies of *Aspergillus* was obtained from the samples. *Aspergillus repens* (67.1%) was the most frequent species and *Aspergillus versicolor*, *Aspergillus candidus* and *Aspergillus amstelodami* were considered to be the common species and their occurrence in the samples was 5.5, 4.6 and 3.2%, respectively.

The species and distribution of the genus *Penicillium* are listed in Table 4. *Penicillium* species were less prevalent than in the *Aspergilli*. *Penicillium canescens* series totaled 27.2% of the *Penicillium* spp. isolated, but its distribution in the samples was not high (1.3%). *Penicillium citrinum*, *Penicillium cyclopium* and *Penicillium notatum* were rocognized as common species.

The genera and distribution of other fungi are listed in Table 5. As was shown in Table 2, *Fusarium*, *Helminthosporium*, *Cladosporium* and *Trichoconis* appeared to occur more frequently than species of *Alternaria* and *Epicoccum*. *Catenularia* was specifically isolated from the Hokkaido samples. It was of interest to note that *Trichoconis* and *Piricularia* have been frequently obtained from these samples. This suggests that most of the samples had been kept under the good storage

Table 3. Species of *Aspergillus* Isolated from Rice in 1965

Species of *Aspergillus*	Total No. of colonies	%	No. of samples from which isolated	%
Aspergillus repens De Bary	286	67.1	19	8.7
A. versicolor (Vuill.) Tiraboschi	22	5.2	12	5.5
A. candidus Link	13	3.1	10	4.6
A. amstelodami (Mangin) Thom	14	3.3	7	3.2
A. ruber (Konig, Spiek., & Brem.) Thom & Church	28	6.6	6	2.7
A. chevalieri (Mangin) Thom & Church	5	1.0	4	1.8
A. mangini Thom and Raper	4	0.9	3	1.4
A. niger v. Tieghem	2	0.5	2	0.9
A. restrictus Smith	31	7.3	1	0.5
A. sclerotiorum Huber	7	1.6	1	0.5
A. penicilloides Spegazzini	2	0.5	1	0.5
A. pseudoglaucus Brochwitz	1	0.2	1	0.5
A. flavus Link	1	0.2	1	0.5
A. flavo-furcutis Batista & Madia	1	0.2	1	0.5
A. avenaceus Smith	1	0.2	1	0.5
A. sydowi (Bain. & Sart.) Thom & Church	1	0.2	1	0.5
A. nidulans (Eidam) Wint	1	0.2	1	0.5
A. terreus Thom	1	0.2	1	0.5
Unidentified species	5	1.0	5	2.3
Total	426			

Table 4. Species of *Penicillium* Isolated from Rice in 1965

Species of *Penicillium*	Total number of colonies	%	No. of samples from which isolated	%
Penicillium citrinum Thom	9	11.7	7	3.20
P. cyclopium Westling	10	14.3	5	2.28
P. notatum Westling	4	5.2	4	1.83
P. canescens series	21	27.3	3	1.37
P. roqueforti Thom	4	5.2	3	1.37
P. citreo-viride Biourge	6	7.8	2	0.91
P. frequentans Westling	3	3.9	2	0.91
P. chrysogenum Thom	2	2.6	2	0.91
P. cyclopium West. var. *echinulatum*	2	2.6	2	0.91
P. expansum Link	2	2.6	2	0.91
P. rugulosum Thom	3	3.9	1	0.46
P. islandicum Sopp	2	2.6	1	0.46
P. sublateritium Biourge	1	1.3	1	0.46
P. decumbens Thom	1	1.3	1	0.46
P. digitatum Saccardo	1	1.3	1	0.46
P. lanoso-coeruleum Thom	1	1.3	1	0.46
P. palitans Westling	1	1.3	1	0.46
P. martensii Biourge	1	1.3	1	0.46
P. tardum Thom	1	1.3	1	0.46
P. sp.	1	1.3	1	0.46
Unidentified species	1	1.3	1	0.46
Total	77			

Table 5. Genera of Fungi Imperfecti and Other Fungi Isolated from Rice in 1965

Genera of Fungi	Total No. of colonies	%	No. of samples from which isolated	%
Fusarium	41	12.3	28	12.8
Helminthosporium	32	9.6	28	12.8
Cladosporium	40	12.0	25	11.4
Phoma	21	6.3	17	7.8
Trichoconis	20	6.0	12	5.5
Catenularia	92	27.7	7	3.2
Piricularia	9	2.7	7	3.2
Alternaria	7	2.1	6	2.7
Chaetomium	6	1.8	4	1.8
Curvularia	7	2.1	3	1.4
Phycomycetous fungi	4	1.2	3	1.4
Coniothyrium	3	0.9	3	1.4
Epicoccum	3	0.9	2	0.9
Nigrospora	2	0.6	2	0.9
Chrysosporium	1	0.3	1	0.5
Doratomyces	1	0.3	1	0.5
Gliomastix	1	0.3	1	0.5
Scopulariopsis	1	0.3	1	0.5
Septoria	1	0.3	1	0.5
Mycelia sterilia	40	12.0	36	16.4
Total	332			

conditions after harvesting and handling.

The results here reported constitute a mycological survey of Japanese milled rice. Most of the samples collected from throughout Japan were invaded by or contaminated with fungi. The fungi comprised mainly species of *Aspergillus*, including *Asp. repens.*, *Asp. versicolor* and *Asp. candidus*. The results of fungal counts showed that the average occurrence of fungi was one to five colonies per 50 grains and 83.2% of the positive samples were in this range. In this respect, Japanese milled rice harvested in 1965 was apparently in favourable hygienic condition. Moreover, all of the samples contained a low percentage of such suspect toxigenic fungi as *Penicillium islandicum*, *P. citrinum*, *P. citreo-viride*, and *Aspergillus flavus*. Japanese milled rice seems to contain a low prevalence of such toxigenic fungi. However, in order to precisely determine the importance of toxigenic fungi associated with Japanese rice, additional investigations of toxigenicity by bioassay techniques will be undertaken in the next phase of this study.

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