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Distribution of Zearalenone-Producing *Fusarium* Species in Japan

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One hundred sixty-six isolates of *Fusarium* spp. from domestic cereal grains, feed, and other sources were examined for their ability to produce zearalenone on autoclaved moist rice grains. They belonged to the following species (number of producers/number tested): *F. roseum* (9/28), *F. roseum* (Culmorum) (3/4), *F. roseum* (Gibbosum) (2/5), *F. roseum* (Avenaceum) (1/2), *F. roseum* (Scirpi) (0/1), *F. tricinctum* (1/4), *F. tricinctum* (Sporotrichiella) (0/7), *F. lateritium* (1/1), *F. episphaeria* (0/2), *F. moniliforme* (0/3), *F. oxysporum* (0/12), *F. rigidiusculum* (0/4), *F. solani* (0/4), *F. splendens* (0/1), *F. nivale* (0/2), and *Fusarium* spp. (15/86). Zearalenone was isolated from molded rice by ethanol extraction and purified by column chromatography. Selected isolates of *F. roseum* M-3-2 and *F. roseum* (Gibbosum) A-O-2 produced 50 to 100 mg of zearalenone per kg of rice. Increased yields (250 to 407 mg/kg of rice) were obtained by *F. roseum* M-3-2 when the substrate was supplemented with 1% peptone.

In previous experiments, toxicological and microbial surveys on trichothec mycotoxins of *Fusarium* spp. were performed in connection with "Akakabi (scabby grain) toxicoses" of men as well as "bean-hull poisonings" of horses and farm animals (14–16). Consequently, nivalenol (11), fusarenon-X (18, 19), T-2 toxin, and neosolaniol (5, 15), all of them having 12-13 epoxy-trichothecene nucleus and potent inhibitors of protein synthesis in animal cells, were detected among the metabolites of *F. nivale*, *F. solani*, *F. sporotrichioides*, and others (17). During the course of this survey, we attempted to clarify the distribution in Japan of fusarial strains that produce zearalenone, a uterotrophic mycotoxin of resorcylic acid lactone (20). We report here an assessment of the wide distribution of the zearalenone producer, environmental factors influencing mycotixon production, and a preparative procedure for isolation of the mycotoxin from moldy rice grains.

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MATERIALS AND METHODS

Sources of cultures. Cultures used in this investigation were isolated from bean hulls, oats, barley, wheat, rice grains, rice straw for bedding in stables, river water, and other sources from the districts where fusarial toxicosis of men and farm animals were sporadically reported (13). Several strains were kindly supplied by H. Kurata (National Institute of Hygienic Sciences), N. Morooka (Kagawa University), and Y. Matsuda (Public Health Research Institute of Koube City). Identification of the species was carried out by one of the authors (H. Tsunoda), according to the taxonomic system of Toussoun and Nelson (12) and that of Bilai (1). All the strains were subcultured on potato-dextrose agar for 14 days at 25 C, followed by storage at 4 C.

Culture of isolates. Inoculum was grown on potato-dextrose agar for 10 days at 25 C, and the aerial mycelia and conidia were seeded into 500-ml Fernbach flasks, each containing 200 g of partly polished rice grains that were previously immersed in tap water for 30 min. After incubation for 14 days at 25 C followed by 14 days at 12 to 15 C (3), the moldy rice was dried at 60 C overnight. Some isolates were also inoculated on the following two liquid media: (i) corn-Czapek medium (glucose, 10 g; sucrose, 20 g; soluble starch, 10 g; $NaNO_3$, 2 g; KCl, 0.5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; corn steep liquor, 10 g; deionized water, 1 liter), and (ii) peptone-supplemented Czapek medium (sucrose, 30 g; $NaNO_3$, 2 g; KCl, 0.5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; peptone, 10 g; and water, 1 liter).

Detection of mycotoxin. Zearalenone was extracted and assayed by the method of Eppley (4) with the following modifications. The laboratory-molded rice powder (50 g) was extracted for 30 min with a

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mixture of 25 ml of water and 250 ml of chloroform in a high-speed blender, and upon filtration the first 50 ml of chloroform extract was charged on a column (2.2 by 30 cm) that consisted of three layers of 5 g of Na_2SO_4, 10 g of silica gel, and again 15 g of Na_2SO_4. The column was eluted with 150 ml of *n*-hexane, 150 ml of benzene, and 250 ml of benzene-acetone (95:5), the last eluate was evaporated to dryness, and the residue was dissolved in 1 ml of benzene. Silica gel (kiesel gel G, Merck) thin-layer plates (0.25 mm, activated for 30 min at 110 C) were spotted with 20 μliters of the benzene solution. The plates were developed with the following solvent systems: benzene-acetic acid (9:1), benzene-acetone (9:1), or chloroform-ethanol (95:5), and inspected under an ultraviolet lamp at 254 nm.

Quantitative analysis. For quantitative analysis, zearalenone was extracted by the Eppley procedure (4) and separated by thin-layer chromatography (Kiesel gel HR, Merck; 0.5 mm in thickness). After development with the solvent *n*-hexane-acetone (4:1), zearalenone-containing gel was scrached from the plates and extracted with methanol, and the content was estimated from the absorption at 236 and 274 nm with a molecular absorption coefficient of 29,700 (236 nm) or 13,900 (274 nm) (20), or an optical density of 0.46 (236 nm) for 5 μg of zearalenon solution per ml.

Instrumental analysis. Melting points were determined with a Mitamura micro-melting-point determinator. Infrared spectra were taken on KBr pellets with a Hitachi model 225 infrared spectrophotometer, and ultraviolet spectra were measured in methanol with a Hitachi model 323 recording spectrophotometer.

RESULTS AND DISCUSSION

Screening for zearalenone producers. Initially, 166 isolates of *Fusarium* were screened for the ability to produce zearalenone when grown on rice grains (Table 1). The mycotoxin was detected from 32 isolates such as *F. roseum*, *F. roseum* (Culmorum), *F. roseum* (Gibbosum), *F. roseum* (Avenaceum), *F. lateritium*, and *F. tricinctum*. These results were the same, except for *F. lateritium*, as those reported by R. W. Caldwell et al. (2). Zearalenone producers were distributed in several districts of Japan and were isolated from oats, barley, wheat, bean hull, and river water (Table 2). Isolates from rice and rice paddies did not produce zearalenone, although rice is an appropriate substrate for zearalenone production.

These findings strongly indicated that the zearalenone-producing fungi are distributed in a wide range of cereal grain as well as feed. This is the first report on the distribution of zearalenone-producing fungi in Japan. In the United States, this mycotoxin has been found in the feed of dairy cattle and is suspected of playing some role in the infertility problems of these animals (8). However, a correlation between the consumption by animals of zearalenone-contaminated scabby grain and abortion and infertility remains to be established (9, 10).

The following isolates, which previously were shown capable of producing trichothecenes (15, 17), also synthesized zearalenone: *Fusarium* sp. M-2-4, *F. roseum* M-3-2, *F. tricinctum* A-B-2, and *F. roseum* (Gibbosum) A-O-2.

Isolation procedure of zearalenone from the moldy rice. Purification and isolation procedures of zearalenone are schematically represented in Fig. 1. *F. roseum* M-3-2 was inoculated on partly polished rice grains, and, after cultivation for 14 days at 25 C followed by 14 days at 12 to 15 C, the moldy rice was dried at 60 C overnight and powdered. The powder (3

TABLE 1. *Zearalenone-producing Fusarium spp.*

Species	No. of producers	No. of isolates
F. roseum	9	28
F. roseum (Culmorum)	3	4
F. roseum (Avenaceum)	1	2
F. roseum (Gibbosum)	2	5
F. roseum (Scirpi)	0	1
F. tricinctum	1	4
F. tricinctum (Sporotrichiella)	0	7
F. lateritium	1	1
F. oxysporum	0	12
F. rigidiusculum	0	4
F. solani	0	4
F. moniliforme	0	3
F. nivale	0	2
F. episphaeria	0	2
F. splendens	0	1
F. spp.	15	86

TABLE 2. *Distribution of zearalenone-producing isolates*

District	Source	No. of producers	No. of isolates
Hokkaido			
Obihiro	Bean hull	4	26
Shizunai	Rice straw	3	6
Abashiri	Barley	2	5
Asahikawa	Oats	2	5
	Rice	0	11
Akita	Paddy	0	2
Fukushima	Bean hull	2	5
Wakayama	River water	3	50
Kagawa	Barley	7	16
	Wheat	3	5
Kouchi	Pimento	0	6
	Melon	0	2
	Vinyl film	0	1
Oita	Barley	0	1
Kumamoto	Barley	0	1
Others		6	24

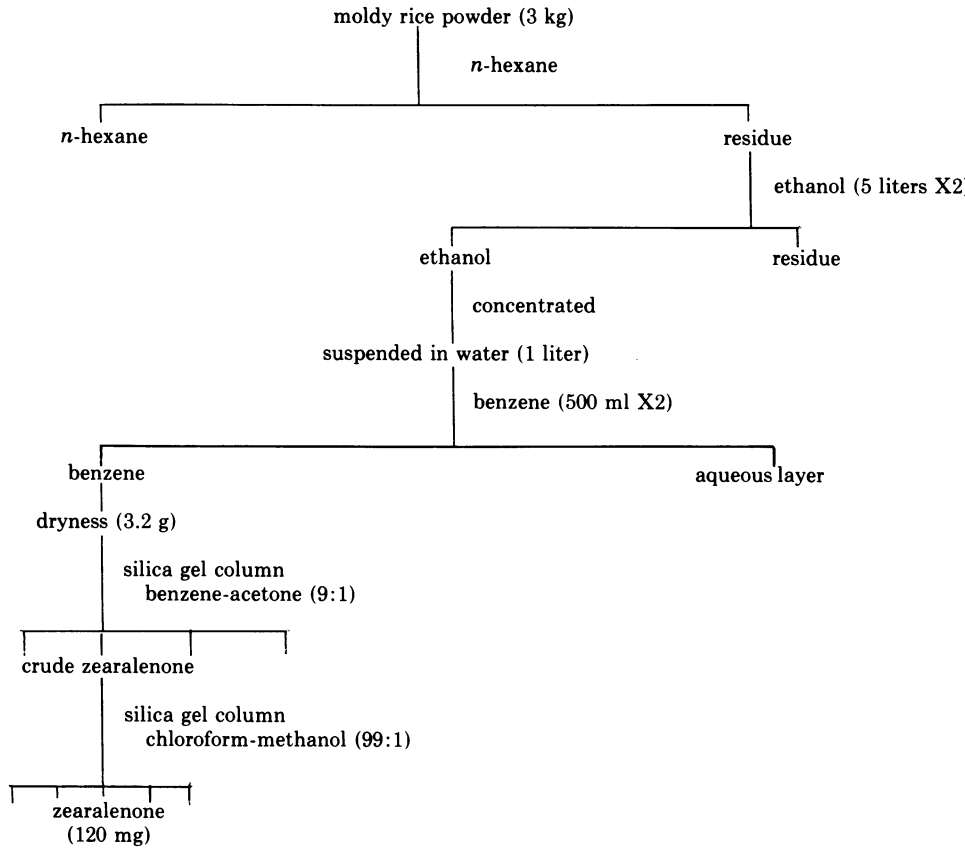

FIG. 1. *Procedure for isolation of zearalenone from moldy rice.*

kg) was continuously extracted with *n*-hexane for 10 to 14 h with a Soxhlet-type extractor, and the defatted residue was extracted twice with 5 liters of ethanol. The combined reddish-yellow ethanol extract was evaporated to a syrup. The residue was suspended in 1 liter of water and then extracted with two 500-ml portions of benzene. Upon evaporation of the solvent, 3.2 g of the oily material was obtained. The first purification of the crude extract on a Kiesel gel column (4 by 45 cm) with 1.5 liter of benzene-acetone (9:1) and the second chromatography with 1 liter of chloroform-methanol (99:1) resulted a yellow powder. Upon crystallization with chloroform and *n*-hexane, white needles were deposited with a yield of 30 to 40 mg/kg of rice (melting point 161 to 162 C; maximal absorbancy 236, 274, and 314 nm). These data and the infrared spectrum (6) confirmed the needles as zearalenone. Under the same conditions and procedures, *F. roseum* Map. 10 (the strain from C. J. Mirocha) produced 50 to 130 mg of zearalenone/kg of rice.

Effect of ingredients on zearalenone production on rice. The effect of supplements on zearalenone synthesis was examined with rice grains as substrate. *F. roseum* M-3-2 was inoculated on rice supplemented with 1% (wt/wt) of various nutrients; after cultivation for 14 days at 25 C followed by 14 days at 15 C, the zearalenone concentration was spectrophotometrically determined at 236 and 274 nm after separation by thin-layer chromatography. The addition of peptone or yeast extract increased the mycotoxin concentration six- and twofold, respectively, over the control (Table 3). Glucose, malt extract, or meat extract was found to suppress the synthesis. Since peptone addition did not result in increased growth, it is presumed that it affected enzyme synthesis required for toxin production. We have no basis for this supposition, for it is conceivable that enzyme derepression could also have occurred.

An additional 14 days of incubation at 25 C after the 12 to 15 C period (7) resulted in increased zearalenone production in both unsupplemented and peptone-supplemented rice (Table 4).

TABLE 3. *Effect of supplements on zearalenone production on rice grain*[a]

Supplements	Content (mg/kg of dried rice)
None	92.0[b]
Glucose	79.2
Peptone	560.0
Yeast extract	160.0
Malt extract	32.0
Meat extract	64.0

[a] *F. roseum* M-3-2 was cultured at 25 C for 14 days and followed at 15 C for 14 days.
[b] Average of duplicate.

TABLE 4. *Production of zearalenone on rice grains in varied culture condition*

Strain	Culture condition	Zearalenone (mg/kg of dried rice)
F. roseum M-3-2	A[a]	40
	B[b]	250
	C[c]	407

[a] Cultured 14 days at 25 C followed by 14 days at 12 to 15 C.
[b] Cultured 14 days at 25 C, 14 days at 12 to 15 C, and then 14 days at 25 C.
[c] The same as B, but 1% peptone was supplemented on rice grains.

Production of zearalenone on liquid media. Zearalenone production was evaluated on two liquid media. *F. roseum* (Gibbosum) A-O-2 was inoculated in 500-ml Fernbach flasks, each containing 300 ml of the culture substrate. After cultivation in standing culture, the culture filtrate was extracted with benzene, and the dried mycelia were first defatted by *n*-hexane followed by extraction with benzene and sequently ethanol. A very small amount of zearalenone was detected by thin-layer chromatography, only in the benzene-soluble extract of mycelia. This lower yield of mycotoxin suggests that the nutritive value of the solid is more conducive to toxin synthesis.

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