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Occurrence of *Gibberella zeae* Strains That Produce Both Nivalenol and Deoxynivalenol

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By single ascospore isolation, several sets of asci containing eight ascospores were isolated from perithecia of *Gibberella zeae*. Of these sets, seven were investigated for their ability to produce 8-ketotrichothecene mycotoxins on rice grains. Analyses were made with gas chromatography-mass spectrometry and gas chromatography with ^{63}Ni electron capture detection. Of 56 total isolates, 11 produced nivalenol, 4-acetylnivalenol, and deoxynivalenol, 1 produced nivalenol and deoxynivalenol, 7 produced deoxynivalenol and 3-acetyldeoxynivalenol, 19 produced deoxynivalenol and 15-acetyldeoxynivalenol, and 6 produced deoxynivalenol and both 15- and 3-acetyldeoxynivalenol. The remaining 12 isolates produced nivalenol and 4-acetylnivalenol. All isolates of *G. zeae* that we examined could produce 8-ketotrichothecenes in this investigation. This report is the first to demonstrate the presence of *G. zeae* isolates producing both nivalenol and deoxynivalenol. In addition, differences in the production between 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol are discussed in relation to culture conditions.

Gibberella zeae (Schw.) Petch (anamorph, *Fusarium graminearum* Schwabe) is a fungal pathogen of wheat, maize, and carnation. This fungus produces many secondary metabolites, including the major mycotoxins nivalenol (NIV), 4-acetylnivalenol (4-AcNIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), and zearalenone (Fig. 1; structure of zearalenone not shown). These mycotoxins can affect the growth and reproduction of livestock; therefore, contamination of cereals and foodstuffs with these trichothecene mycotoxins is of great concern to food scientists (26–28). NIV and DON have been frequently detected in cereals harvested in more than 20 countries and districts (12, 24, 25). The acute toxicity of NIV is several times higher than that of DON (20), and a long-term feeding of NIV results in aleukia and malnutrition in mice (20, 29). However, the tumorigenic potential was negative in mice fed NIV-containing diets for 2 years (18).

In a previous report, we noted that *G. zeae* is chemotaxonomically divided into two types; one is the NIV chemotype, which produces NIV and 4-AcNIV; and the other is the DON chemotype, which produces DON and 3-AcDON (11). Logrieco et al. (15) have also reported these two chemotypes in cereals harvested in southern Italy. No cross-production of these two types of trichothecenes was observed in *G. zeae*. The presence of the *G. zeae* NIV chemotype was reported in Japan, Korea, Taiwan, and Italy (1, 11, 13, 15); however, no reports were found from Canada, the United States, and South Africa, although grain contaminated with NIV was reported in these countries (22, 24, 25). In addition, little is known about the mycological differences between these two chemotypes of *G. zeae*. Therefore, we have embarked on research attempting to increase our understanding of the population and distribution of these two chemotypes in the field. Using efficient extraction and sensitive detection methods, we studied the ability of *G. zeae* to produce 8-ketotrichothecenes and the pattern of main products of its progeny in the field.

MATERIALS AND METHODS

Sampling for *G. zeae*. In June 1988, perithecia of *G. zeae* formed on rice stubble were collected from barley and wheat fields in five different locations in Japan: (i) Kogota-Shi (TK) and (ii) Taiwa-Shi (TY), Miyagi Prefecture; (iii) Mizusawa-Shi (MM), Iwate Prefecture; (iv) Hirosaki-Shi (TH), Aomori Prefecture; and (v) Kounan-Cho (SC), Saitama Prefecture. Samples collected were stored at 4°C in paper bags.

Dissection of asci. A piece of rice stubble with perithecia was placed on the stage under a stereomicroscope (×30). A single perithecium was picked up with sterile forceps and then placed in a drop of sterile water on the surface of a 5% (wt/vol) water agar sheet in a small disposable petri dish (52 by 11 mm). Individual asci were separated from the main cluster with sterile forceps, and eight ascospores were successively pulled from the tip of the ascus with a Skerman micromanipulator (21) (Toyo Rikoki Co. Ltd., Tokyo). Each ascospore was dragged on the plate for a short distance to avoid contaminants, such as bacteria and other fungi. Small squares of the agar each containing a single ascospore were cut out with a dissecting needle under the stereomicroscope (×50) and transferred to a potato-dextrose agar (PDA) slant. Each set of eight ascospores was cultured at 25°C for 10 days and then stored at 4°C until examined.

Analysis of trichothecene mycotoxins. Samples of 20 g of polished rice grains (ca. 33% moisture content) in 300-ml Erlenmeyer flasks were autoclaved for 20 min at 120°C, and then 5 ml of a sterile 3% peptone (Bacto-Peptone; Difco Laboratories, Detroit, Mich.) solution was added. The inoculum for each flask was a plug (21 mm in diameter) from a PDA culture grown at 25°C for 7 days. The cultures were incubated in the dark at 25°C for 21 days.

Trichothecenes in the moldy rice grains were directly extracted with acetonitrile-water (3:1, vol/vol), and the extracts were evaporated to dryness under vacuum by adding

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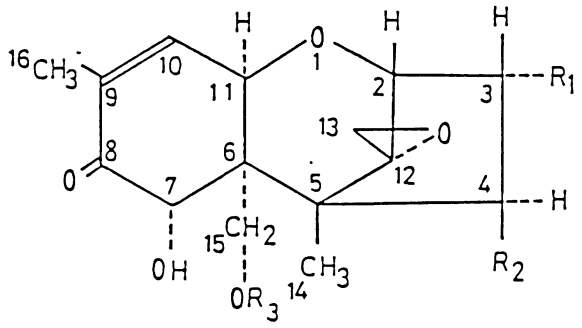

	R_1	R_2	R_3
4-Acetylnivalenol	OH	OAc	H
Nivalenol	OH	OH	H
3-Acetyldeoxynivalenol	OAc	H	H
15-Acetyldeoxynivalenol	OH	H	OAc
Deoxynivalenol	OH	H	H

FIG. 1. Structures of 8-ketotrichothecene mycotoxins.

an equal volume of ethanol. The residue was dissolved in 4 ml of methanol and added to a column (30 by 2.2 cm) of Florisil packed in chloroform. Trichothecenes were eluted with 100 ml of chloroform-methanol (9:1, vol/vol), and the eluate was evaporated to dryness (23). The residue was reacted with trimethylsilylating reagent (Gasukuro Kogyo Inc., Tokyo), which contained *N*-trimethylsilylimidazole–trimethylchlorosilane–ethyl acetate (1:0.2:9). Gas chromatography-mass spectrometry with selected ion monitoring mode and full mass spectrum analysis was performed for quantitative estimation and confirmation of the trichothecenes. Fragment ions monitored were *m/z* 512 and 422 for DON, *m/z* 510 and 379 for NIV, *m/z* 480 for 4-AcNIV, *m/z* 377 for 3-AcDON, and *m/z* 392 and 350 for 15-AcDON. In addition, gas chromatography with ^{63}Ni electron capture detection was performed to support the quantitative estimation by gas chromatography-mass spectrometry. The detection limits for 8-ketotrichothecenes by gas chromatography-mass spectrometry and gas chromatography with ^{63}Ni electron capture detection were 10 and 2 ng/g, respectively (23).

Reagents. Standard NIV, 4-AcNIV, DON, and 3-AcDON were prepared in our laboratory. The 15-AcDON standard was supplied by C. J. Mirocha, University of Minnesota. All organic solvents used were of analytical reagent grade. Florisil PR (60–100 mesh; Wako Pure Chemicals, Osaka) was activated at 130°C for 2 h before use.

RESULTS

Germination of ascospores isolated from an ascus. Of the 13 mature asci examined, 12 contained eight ascospores; 98 of the 103 ascospores derived from these asci germinated. There was no more than one nongerminant ascospore per ascus. Ascospores without septa or less than one-half the normal size generally did not germinate. During these isolations we made several interesting observations: (i) one or two ascospores per ascus began to germinate after approximately 2 h; (ii) the remaining ascospores germinated within 24 h; (iii) germ tubes developed from the two terminal cells of the ascospore (as observed by Headrick et al. [9]); and (iv) ascus walls of *G. zeae* were elastic, and the ascus length could be doubled with a microhook.

Production of 8-ketotrichothecenes. Table 1 shows the production of 8-ketotrichothecenes by seven sets of the ascospores of *G. zeae*. Since the eight ascospores of *G. zeae* asci are not in linear order, the numbered strains in Table 1 do not coincide with arrangement of ascospores in the ascus. On the basis of differences in the main products, these seven sets were subdivided into two groups—the DON group and the NIV group. In the DON group, all TK-1 strains produced DON and 3-AcDON, along with 15-AcDON in two strains (TK-1-2 and -6). In contrast, all TK-2 strains, which were derived from perithecium samples from different rice stubble in the same field, produced only DON and 15-AcDON. Among TH-4 strains, one strain (TH-4-5) produced DON and 3-AcDON, three strains (TH-4-1, -4, and -6) produced DON and 15-AcDON, and the remaining four strains (TH-4-2, -3, -7, and -8) produced DON, 3-AcDON, and 15-AcDON. In TH-5 strains, which were derived from the same perithecium, all strains produced DON and 15-AcDON. In the NIV group, all TY-1 strains produced both NIV and 4-AcNIV except TY-1-8, which lacked 4-AcNIV. They also produced low levels of DON. In SC-1 and MM-1 strains, all eight strains produced NIV and 4-AcNIV, whereas two strains produced lesser amounts of DON. All of the strains we examined could produce at least one 8-ketotrichothecene.

Since TY-1 strains produced small amounts of DON, along with the major product, NIV (Table 1), two isolates (TY-1-1 and -3) were selected and cultured every 7 days for 21 days to confirm DON formation. The results revealed that the production of NIV and 4-AcNIV increased with incubation time, whereas a low level of DON was detected during the entire culture period (data not shown).

In addition, we studied the effects of environmental factors, particularly temperature, on the synthesis of 3-AcDON and 15-AcDON. When two sets of the DON chemotype (TK-1 and TH-4) were cultured at 25°C for 2 weeks followed by 4°C for 2 weeks, they produced only 15-AcDON along with the major product DON, and no 3-AcDON was detected (Table 2).

DISCUSSION

In this experiment, we examined the progeny to characterize the production of 8-ketotrichothecenes of *G. zeae* from a population genetics point of view. Of seven sets, four contained progeny that produced DON and lesser amounts of its 3- and/or 15-acetyl derivatives. The progeny from the other three sets primarily produced NIV (Table 1). These results confirmed our previous finding that *G. zeae* isolates can be divided into DON and NIV chemotypes (11). However, of 24 strains in the NIV chemotype, 12 could also produce DON at a low level (Table 1). This is the first report demonstrating production of both NIV and DON by *G. zeae* isolates from nature.

As for the DON chemotype, we found six strains that produced both 3-AcDON and 15-AcDON (Table 1). A strain that produced both esters was also reported in the United States (16). Other workers (10, 31), however, suggested that 3-AcDON and 15-AcDON were produced independently by different DON-producing strains of *F. graminearum* and, therefore, that DON-producing strains (DON chemotype) could be subdivided into two types with respect to the production of DON esters. Our results suggest that 3- and

TABLE 1. Production of 8-ketotrichothecenes by *G. zeae* strains

Group and strain	8-Keto-trichothecene	Production (μg/g) of 8-ketotrichothecene by individual ascospores from an ascus							
		1	2	3	4	5	6	7	8
DON group									
TK-1	NIV	ND[a]	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	53.83	28.59	34.81	43.37	36.70	33.07	29.88	24.55
	3-AcDON	3.54	1.57	2.41	2.00	2.94	2.34	1.46	1.53
	15-AcDON	ND	1.13	ND	ND	ND	2.72	ND	ND
TK-2	NIV	ND	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	3.69	4.06	3.08	5.17	4.04	2.10	3.31	2.65
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	1.75	1.37	1.57	1.76	1.27	1.03	1.49	0.69
TH-4	NIV	ND	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	22.36	26.43	18.73	17.98	48.27	12.20	32.98	19.38
	3-AcDON	ND	1.69	1.80	ND	2.16	ND	2.07	1.86
	15-AcDON	4.82	1.07	2.91	9.84	ND	4.18	1.25	2.12
TH-5	NIV	ND	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	186.52	203.64	76.66	160.52	272.18	127.46	366.97	146.90
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	11.84	12.66	5.23	8.96	26.68	11.30	64.17	9.45
NIV group									
TY-1	NIV	15.52	4.77	102.43	13.06	11.54	6.41	12.86	0.97
	4-AcNIV	5.78	2.13	15.70	5.27	5.43	2.41	5.56	ND
	DON	0.32	0.09	0.74	0.45	0.21	0.09	0.43	0.01
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
MM-1	NIV	0.75	0.66	0.75	0.24	0.18	0.06	0.87	0.60
	4-AcNIV	0.11	0.10	0.23	0.05	0.03	0.04	0.05	0.26
	DON	0.02	0.01	ND	ND	ND	ND	ND	ND
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
SC-1	NIV	0.57	0.20	0.69	0.69	0.34	1.53	0.66	0.62
	4-AcNIV	0.16	0.01	0.11	0.10	0.05	0.30	0.25	0.04
	DON	ND	ND	0.02	ND	0.01	ND	ND	ND
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND, Not detected.

15-acetylation of DON are dependent on environmental temperature, and that the formation of 15-AcDON by 3-AcDON-producing strains is presumed to be caused by an expression of an enzymatic activity that catalyzes the conversion of 3-AcDON into 15-AcDON. Therefore, in our opinion differences in the production of 3-AcDON or 15-AcDON are not chemotaxonomic characteristics of *G. zeae*. Further studies on the relationship between the acetylation of DON and culture temperature are needed.

From our results mentioned above, we correct our previous opinion as follows: *G. zeae* strains of the NIV chemotype produce NIV and 4-AcNIV and may also produce trace or low levels of DON, whereas strains of the DON chemotype produce DON and its acetates (3-AcDON, 15-AcDON, or both) but do not produce NIV or its acetates. Currently, it has been demonstrated that some diacetoxyscirpenol-producing *Fusarium* sp. can transform DON to NIV and its acetates (3). According to Yoshizawa (30), an NIV-producing strain of *F. graminearum* converted DON into NIV and its acetates. However, there are no reports of esterification at the C-4 position by strains belonging to the DON chemotype of *G. zeae* (*F. graminearum*). Therefore, an understanding of the enzymatic or genetic regulation of microbial modifications at C-4, rather than C-3 or C-15, is necessary to clarify the difference between two chemotypes. *G. zeae* is usually considered to be homothallic ascomycete (8, 17). Although heterothallic strains (unable to form perithecia on carnation leaf agar medium) have been reported (4, 6), there are no reports of heterothallism based on sexual reproduction by crosses between two strains with different mating types. Among species of *Gibberella*, *G. pulicaris* and *G. fujikuroi* are heterothallic; therefore, the regulation mechanism has been easily approached by crossing two different mating types with selective markers (7, 19). *G. zeae* is homothallic, vegetatively incompatible, and lacks a parasexual cycle. Thus, it is hard to carry out the genetic analysis by crosses in this fungus. To solve this difficulty, the technique of protoplast fusion was recently introduced (2, 5, 14). Studies with this protoplast fusion technique are under way to define genetic differences between these two chemotypes.

TABLE 2. Production of 8-ketotrichothecenes with TK-1 and TH-4 strains after the incubation at 25°C for 14 days followed by 4°C for 14 days

Strain	8-Keto-trichothecene	Production (μg/g) of 8-ketotrichothecene by individual ascospores from an ascus							
		1	2	3	4	5	6	7	8
TK-1	NIV	ND[a]	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	27.79	26.90	40.75	42.86	10.77	38.31	12.57	30.82
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	9.37	2.76	5.35	4.06	3.72	5.92	9.27	5.44
TH-4	NIV	ND	ND	ND	ND	ND	ND	ND	ND
	4-AcNIV	ND	ND	ND	ND	ND	ND	ND	ND
	DON	13.16	13.68	7.40	19.95	10.62	23.45	21.51	9.50
	3-AcDON	ND	ND	ND	ND	ND	ND	ND	ND
	15-AcDON	7.82	7.36	2.98	4.87	5.89	4.85	3.20	5.44

[a] ND, Not detected.

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