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Fusarium crookwellense, a newly isolated fungus from wheat in Japan: Its mycotoxin production and pathogenicity to wheat and barley

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Fusarium crookwellense **was isolated for the first time in Japan from scabby wheat harvested in Hokkaido in 1991. Four isolates were obtained and examined for their mycological features on culture media, mycotoxin production, and pathogenicity to wheat and barley. The texture of fungal colonies and the morphology of macroconidia on a potato dextrose agar medium were similar to those of *Fusarium graminearum*. All *F. crookwellense* isolates examined produced nivalenol, 4-acetylnivalenol, and zearalenone on rice media at levels ranging from 0.9 to 22.5 μg/g, 0.5 to 25.0 μg/g, and 1.4 to 162.5 μg/g, respectively. All were pathogenic toward the wheat and barley strains tested, and scab symptoms were found on the heads and leaves of plants. This is the first report on domestic isolates of *F. crookwellense* from the crop field in Japan.**

Key Words——4-acetylnivalenol; *Fusarium crookwellense*; nivalenol; pathogenicity; zearalenone.

Introduction

Several species of the genus *Fusarium* possess potent pathogenicity toward important cereal crops such as wheat, barley and maize, and produce toxic secondary metabolites in cereals. The contamination by these chemicals of foods and feeds is closely associated with the outbreak of food-born diseases in humans and livestock, as reviewed by one of the authors (Ueno, 1986, 1987). We have demonstrated the worldwide occurrence of toxic metabolites such as the trichothecenes, nivalenol (NIV) and deoxynivalenol (DON), and a phytoestrogen, zearalenone (ZEN), in cereals and food products (Tanaka and Ueno, 1989). These mycotoxins are major products of *Fusarium graminearum* Schwabe (teleomorph, *Gibberella zeae* (Schw.) Petch), which is an important pathogen and responsible for scab disease, "Akakabi-byo", in Japan.

Fusarium crookwellense Burgess, Nelson & Toussoun was first isolated from a potato tuber at Crookwell in Australia in 1971, and was classified as a new species of *Fusarium* (Burgess et al., 1982). Its presence has hitherto been reported in Australia, Canada, China, Colombia, Denmark, Finland, France, Germany, Italy, New Zealand, Poland, South Africa, the United States, and Yugoslavia (Golinski et al., 1988; Bottalico et al., 1990; Thrane, 1990, Miller et al., 1991; Lauren et al., 1992). Several metabolites such as NIV, 4-acetylnivalenol (4-AcNIV), ZENs, butenolide, and culmorin are reported to be the toxic chemicals (Golinski et al., 1988; Frisvad and Samson, 1991). In Japan, the occurrence of *F. crookwellense* has not been reported despite several studies on the distribution of scab fungi on wheat and barley (Ichinoe et al., 1983; Koizumi et al., 1991).

In our surveys of the distribution of NIV-producing *Fusarium* species in Hokkaido, isolates of *F. crookwellense* were obtained for the first time from wheat sampled in a field in western Hokkaido. The present paper describes the morphology, mycotoxin production, and pathogenicity of domestic *F. crookwellense* isolates.

Materials and Methods

Isolation On 19 July 1991, scab heads of wheat were collected in a field in Koshin, Kyogoku-Cho (Shiribeshi district) in Hokkaido. After sampling, wheat grains were surface-sterilized in a 0.5% aqueous sodium hypochlorite solution for 1 min, then rinsed three times with sterile water. In each of 10 petri dishes, five grains were plated onto potato dextrose agar (PDA) (Difco) containing 100 μg of chloramphenicol per ml. After incubation at 25°C for 1 week, dark red or crimson fungal colonies developed. After single spore isolation, four isolates were obtained and identified as *F. crookwellense* according to

the *Fusarium* taxonomic system of Nelson et al. (1983) and the original description (Burgess et al., 1982). The isolates were cultured on PDA and stored at 4°C until examination. Of these *F. crookwellense* isolates, strains KH-1-3, KH-2-1, and KH-4-5 were maintained as IFO 32584, 32585, and 32586, respectively, at the Institute for Fermentation, Osaka.

Strain For comparison with the *F. crookwellense* isolates, *F. culmorum* KF-98, and *F. graminearum* TH-5-1 and KF-208 (for photograph) were used from the collection of our laboratory at the Science University of Tokyo. Strain ARC 2132 of *F. graminearum* was obtained from the National Agriculture Research Center (Tsukuba).

Analysis for mycotoxis Mycotoxins were examined by our previous method (Tanaka et al., 1985a). Briefly, after incubation at 25°C for 2 weeks, moldy rice grains were dried in a hot-air oven at 45°C for 40 h and finely powdered in a blender. The samples were extracted with acetonitrile-water (3 : 1, vol/vol), and the extracts were evaporated to dryness under vacuum. The toxins were purified by column chromatography on Florisil. After derivatization by trimethylsilylating reagent (Gasukuro Kogyo, Tokyo), which contained *N*-trimethylsilylimidazole-trimethylchlorosilane-ethyl acetate (1 : 0.2 : 9), the amounts of seven trichothecenes (DON, 3-acetyldeoxynivalenol (3-AcDON), NIV, 4-AcNIV, diacetoxyscirpenol (DAS), neosolaniol (NS) and T-2 toxin (T-2)) and ZEN were estimated by gas chromatography-mass spectrometry (Hitachi Model M-80A, Hitachi, Tokyo) with selected ion monitoring. The detection limits were 0.05 μg/g for DON, 3-AcDON, NIV and 4-AcNIV, and 0.1 μg/g for T-2, DAS, NS and ZEN.

Pathogenicity to wheat and barley Three strains of cereals, cv. Kashimamugi (barley), cvs. Fujimikomugi and Gabo (wheat), were used to examine the pathogenicity of *F. crookwellense* isolates. After seeding in a seedling case (4×8×8 cm), the barley and the wheats were cultured respectively for 40 days and 55 to 60 days in a greenhouse. When the plants reached the flowering stage, a spore suspension of a *F. crookwellense* isolate was sprayed onto the heads of the plants. The spore suspension contained ca. 50 spores in 150× microscope field and was prepared from a potato extract broth that had been incubated for 5 days at 25°C with shaking. *F. graminearum* ARC 2132 was examined as a positive control. To promote the germination of macroconidia after spore inoculation, the plants were stood for 24 h at 25°C in a moist chamber with high humidity (100% moisture), then transferred to the greenhouse and cultured until symptoms were observed.

Results

Fusarium crookwellense Burgess, Nelson & Toussoun
Figs. 1-2

Cultures on PDA after 5 days were livid red to crimson, developing brown to dark brown sporodochia in the center and sometimes at the edge and on the wall of the petri dish. Growth rate in 1 week was 64 mm at 25°C, 38 mm at 15°C, and 10 mm at 4°C, but zero at 35°C. Aerial mycelium was floccose, white, red and yellow-brown. Macroconidia were produced abundantly on the medium, measuring 24-62×(4-)5-7 μm (5 septate) or 28-58×5-7 μm (4 septate). Macroconidia were formed from monophialides or from branched conidiophores which terminated in short monophialides. Microconidia, polyblastic and polyphialidic cells were not observed. Chlamydospores were not also observed during 3 weeks of incubation. Culture on a carnation leaf agar (CLA)

Table 1. Measurement of macroconidia of *F. crookwellense* isolates and related species on potato dextrose agar medium.

Strain	Septate	No. of conidia measured	Range	Mean
			μm	μm
F. crookwellense	5	20	40−54×(4−)5−6	46.2×5.4
KH-1-3	4	10	38−46×5−6	43.1×5.2
	3	3	36−48×5−6	42.7×5.3
KH-2-1	5	20	42−50×5−6	45.1×5.5
	4	10	40−50×5−7	43.4×6.1
KH-3-4	6	7	46−68×5−7	54.0×5.9
	5	15	48−62×5−7	53.2×5.7
	4	10	44−58×5−6	48.8×5.4
KH-4-5	5	20	24−48×5−7	38.1×5.6
	4	17	28−38×6−7	32.1×6.3
	3	10	24−34×5−7	28.6×5.8
F. graminearum	5	20	36−50×5−6	43.9×5.5
TH-5-1	4	15	32−56×4−6	38.6×4.7
	3	10	26−38×4−5	30.6×4.3
F. culmorum	5	20	28−44×5−7	36.3×6.2
KF-98	4	10	26−36×4−7	32.4×5.7
	3	15	24−30×4−6	27.0×4.7

medium formed abundant pale orange to dark brown sporodochia on the leaves. The macroconidia were typical spore types, which were usually 5 septate, thick-walled, widest at the central part, arched and with curved dorsal rather than ventral surface, with the basal cell distinctly foot-shaped and the apical cell curved and tapering to a narrow tip. Size of macroconidia was 40–56×(4–)5–7 μm (5 septate) or 38–50×(4–)5–7 μm (4

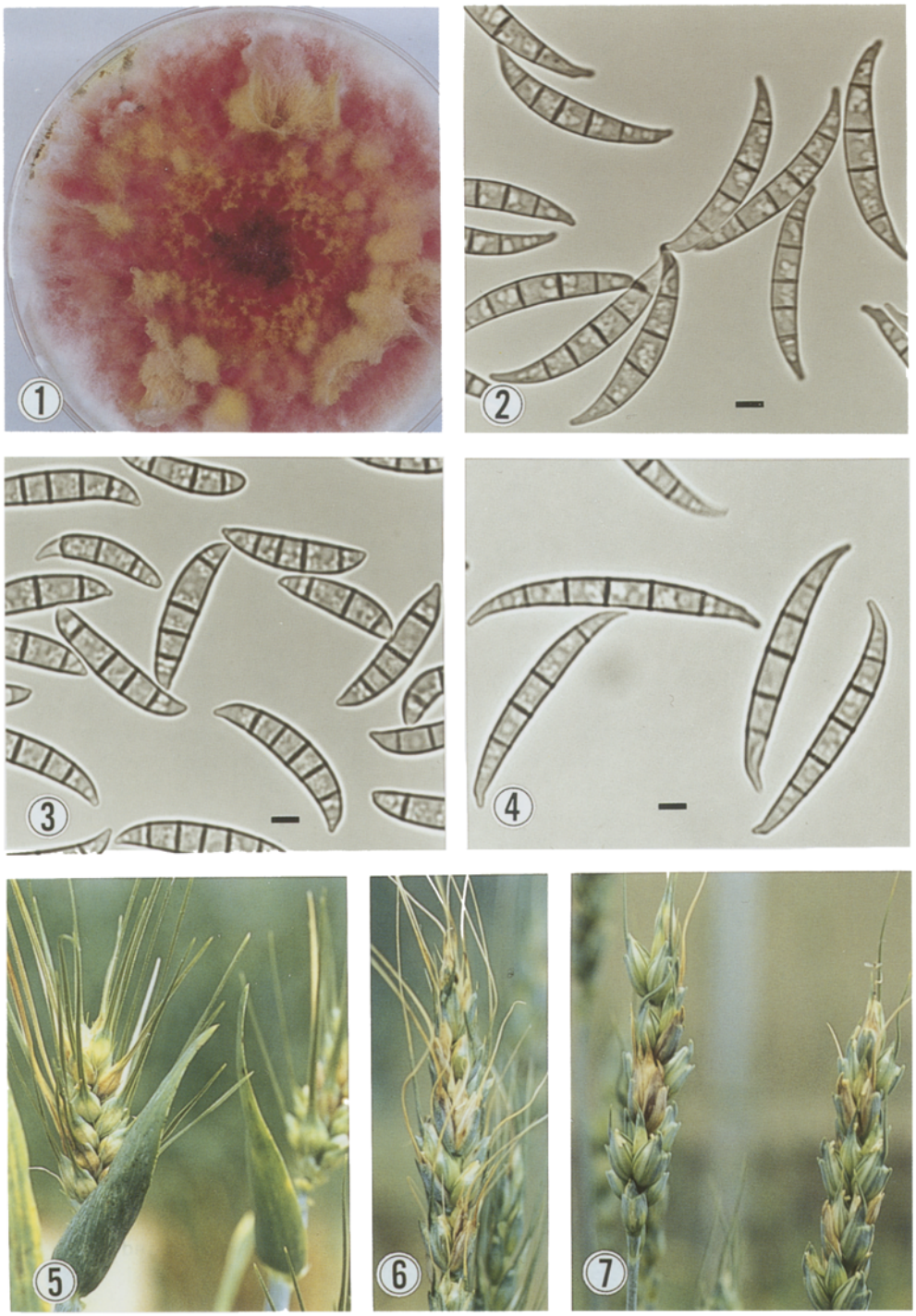

Fig. 1. Colony of *Fusarium crookwellense* (KH-2-1) on PDA medium after 1 week.
Fig. 2. Macroconidia of *F. crookwellense* (KH-2-1) formed on CLA medium.
Fig. 3. Macroconidia of *F. culmorum* (KF-98) on CLA medium.
Fig. 4. Macroconidia of *F. graminearum* (KF-208) on CLA medium (Scale bars=6 μm in Figs. 2–4.).
Figs. 5–7. Disease symptoms on heads of wheat and barley infected by *F. crookwellense*. 5. cv. Kashimamugi (barley) cultivated in greenhouse. 6. cv. Fujimikomugi (wheat) in greenhouse. 7. cv. Gabo (wheat) in greenhouse.

septate). Formation of perithecium was not observed on the CLA medium during 6 weeks of incubation.

During initial growth, the macroconidia of *F. crookwellense* formed on PDA medium were morphologically similar to those of *Fusarium culmorum* (Fig. 3). The macroconidia were slightly wide, and the apical cell was slightly blunt. Thereafter, it resembled *F. graminearum* (Fig. 4). Although the macroconidia of *F. crookwellense* on PDA medium were usually 4 or 5 septate, 3 and 6 septate conidia were also observed in smaller numbers (Table 1). On CLA medium, the macroconidia of *F. crookwellense* were more arched in the dorsal surface than those of *F. graminearum*, and had a distinct foot-shaped base to the basal cell and a tapering apical cell. Therefore, *F. crookwellense* was distinguished from *F. graminearum* and *F. culmorum* by the shape of the macroconidium. In rare cases, macroconidia with 2 or 3 septa were found on CLA medium. Other media such as Czapek Dox agar (CZ), corn meal agar, and malt extract agar (MA) were not suitable for the identification of *F. crookwellense*. In particular, CZ and MA media gave poor or irregular (2-3 septate) formation of macroconidia.

Mycotoxin production As summarized in Table 3, all four isolates of *F. crookwellense* produced NIV, 4-AcNIV, and ZEN at levels ranging from 0.9 to 22.5 μg/g, 0.5 to 25.0 μg/g, and 1.4 to 162.5 μg/g, respectively. Among the isolates, strain KH-2-1 gave high amounts of NIV, 4-AcNIV, and ZEN at levels of 22.5, 25, and 162.5 μg/g, respectively. Other trichothecenes such as DON, 3-AcDON, DAS, NS, and T-2 were below the detection limit (<0.05 μg/g for DON and 3-AcDON, and 0.1 μg/g for DAS, NS, and T-2).

Pathogenicity All four *F. crookwellense* isolates were found to be virulent to cv. Kashimamugi, Fujimikomugi, and Gabo within 15 days after spore inoculation (Table 4). Strain ARC 2132 of *F. graminearum* developed scab symptoms within 7 days. Symptoms of *F. crookwellense* infection were mainly observed on the heads of test plants (Figs. 5-7) and were identified as scab. Among the three plants, cv. Fujimikomugi was most susceptible to infection by the *F. crookwellense* isolates examined. Strains KH-2-1 and KH-3-4 developed symptoms on both heads and leaves of this plant and produced sporodochia on the surface of wheat grains obtained after 55 days of cultivation.

Discussion

Based on the morphology of macroconidia, *F. crookwellense* was described as a new species belonging to section Discolor (Burgess et al., 1982). Subsequently, Nirenberg (1990) recognized it as being identical with *Fusarium cerealis* (Cooke) Sacc. which was known prior to 1982. In the present paper, we examined the mycological features on culture media, mycotoxin production, and the pathogenicity of *F. crookwellense* isolated in Hokkaido, based on the taxonomic system of Nelson et al. (1983). Burgess et al. (1982) reported that *F. crookwellense* is distributed in mild climatic areas of moderate rainfall and is absent from areas of extreme temperature. Although Hokkaido is the coldest region in Japan and has low rainfall in summer, its climate is temperate with moderate rainfall. Thus, the occurrence of *F. crookwellense* in Japan agrees with previous reports.

The macroconidia of *F. crookwellense* grown on PDA medium are morphologically similar to those of *F. graminearum* as previously pointed out (Burgess et al., 1982). At the beginning of culture, it resembled *F. culmorum* in the morphology and range of the conidia. Of the four isolates, strain KH-4-5 was similar to *F. culmorum* in the size of macroconidia (Table 1). Thus, the identification of *F. crookwellense* based on the shape of macroconidia formed on PDA medium may lead to confusion with *F. graminearum* or *F. culmorum*. To differentiate *F. crookwellense* from *F. graminearum* and *F. culmorum*, CLA was a useful medium. The macroconidia formed on carnation leaves were uniform, and showed the typical spore type of *F. crookwellense* (Fig. 2, Table 2).

Table 3 showed that *F. crookwellense* isolates produced two derivatives of trichothecenes, NIV and 4-AcNIV, and ZEN. These results agree with previous works (Golinski et al., 1988, Lauren et al., 1992). We have previously reported that wheat grains harvested from several areas in Hokkaido were positive for DON, NIV, and ZEN (Tanaka et al., 1985b), and that mycological surveys demonstrated that *F. graminearum* isolates derived from Hokkaido produced DON, and *F. poae* isolates produced NIV (Tanaka et al., 1987). In addition, scabby wheat grains sampled from a field in Kyogoku, where the present *F. crookwellense* was isolated, were positive for NIV with a significant level of 1.22 μg/g (Sugiura et al., 1993). Thus, it is possible that wheat grains infected by *F. crookwellense* may be contaminated with NIV. Several strains of *F. crookwellense* have also been isolated from maize kernels harvested in Memuro, located near Obihiro (Tokachi district) (unpublished data). Thus, *F. crookwellense* appears to be distributed in crop fields throughout Hokkaido and to be responsible for the natural contamination with NIV found in cereals in Hokkaido. As a limited number of *F. crookwellense* isolates was examined, further surveys are required to clarify the relationship between the distribution of *F. crookwellense* and the occurrence of NIV contamination in Hokkaido.

In Hokkaido, *F. graminearum, F. avenaceum, F. culmorum* and *Microdochium nivale* are responsible for scab disease in wheat and barley (Koizumi et al., 1991). The *F. crookwellense* isolates also developed scab symptoms on heads of wheats and barley tested (Figs. 5-7), which were identical with those of strain ARC 2132 of *F. graminearum* examined as a positive control, although the incubation period required for development of symptoms by the isolates was twice as long as that of strain ARC 2132 (Table 4). Thus, it was demonstrated that these *F. crookwellense* isolates are responsible for scab disease in wheat and barley. Since *F. crookwellense* and *F. graminearum* resemble each other, further studies are required to clarify the difference in incubation period between these two *Fusarium* species.

In conclusion, *F. crookwellense* was isolated for the

Table 2. Measurement of macroconidia of *F. crookwellense* isolates and related species on carnation leaf agar medium.

Strain	Septate	No. of conidia measured	Range	Mean
			μm	μm
F. crookwellense	5	20	40−50×5−7	44.8×6.0
KH-1-3	4	10	40−42×5−6	41.0×5.8
KH-2-1	5	25	42−52×5−7	48.1×5.9
	4	18	38−50×(4−)5−6	44.6×5.6
KH-3-4	5	20	44−56×5−7	49.5×6.2
	4	16	42−48×5−7	45.6×5.9
KH-4-5	5	25	40−50×(4−)5−7	45.3×6.0
	4	15	40−48×5−7	43.7×6.0
F. graminearum	5	20	38−54×4−6	47.4×5.2
TH-5-1	4	18	36−52×4−6	41.5×4.9
	3	12	30−42×4−5	35.0×4.4
F. culmorum	5	20	24−46×6−8	40.5×6.4
KF-98	4	16	28−44×6−7	35.9×6.4
	3	15	28−38×5−8	31.9×5.6

Table 3. Production of mycotoxins by *Fusarium crookwellense*.

Strain	*Mycotoxins (μg/g)							
	NIV	4-AcNIV	DON	3-AcDON	DAS	NS	T-2	ZEN
KH-1-3	7.3	10.9	**ND	ND	ND	ND	ND	5.0
KH-2-1	22.5	25.0	ND	ND	ND	ND	ND	162.5
KH-3-4	5.4	4.1	ND	ND	ND	ND	ND	2.9
KH-4-5	0.9	0.5	ND	ND	ND	ND	ND	1.4

*NIV: nivalenol; 4-AcNIV: 4-acetylnivalenol; DON: deoxynivalenol; 3-AcDON: 3-acetyldeoxynivalenol; DAS: diacetoxyscirpenol; NS: neosolaniol; T-2: T-2 toxin; ZEN: zearalenone
**ND: not detected

Table 4. Pathogenicity of *Fusarium crookwellense* isolates and *F. graminearum* to wheat and barley.

Test plant	Cultivar	Strain		Disease	
				*Symptom	**Day
Barley	Kashimamugi	*F. crookwellense*	KH-1-3	+	11
			KH-2-1	+	11
			KH-3-4	+	11
			KH-4-5	+	11
		F. graminearum	ARC2132	+	7
Wheat	Fujimikomugi	*F. crookwellense*	KH-1-3	+	12
			KH-2-1	++	12
			KH-3-4	++	12
			KH-4-5	+	12
		F. graminearum	ARC2132	+	7
	Gabo	*F. crookwellense*	KH-1-3	+	15
			KH-2-1	+	15
			KH-3-4	+	15
			KH-4-5	+	15
		F. graminearum	ARC2132	+	7

*++: symptoms on heads and leaves; +: symptoms on heads alone.
**The day after inoculation on which scab symptoms were observed.

first time in Japan from scabby wheat. Its morphological characteristics on PDA medium were similar to *F. graminearum* and sometimes *F. culmorum*. Therefore, it was possible that *F. crookwellense* may be misidentified as *F. graminearum* or *F. culmorum*. Since *F. crookwellense* isolates examined produced NIV and possessed pathogenicity to wheat and barley, it was proposed that *F. crookwellense* is responsible for the NIV contamination of wheat and scab disease in Hokkaido.

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Literature cited

Bottalico, A., Logrieco, A. and Visconti, A. 1990. Mycotoxins produced by *Fusarium crookwellense*. Phytopath. Medit. **29**: 124–127.

Burgess, L. W., Nelson, P. E. and Toussoun, T. A. 1982. Characterization, geographic distribution and ecology of *Fusarium crookwellense* sp. nov. Trans. Br. Mycol. Soc. **79**: 497–505.

Frisvad, J. C. and Samson, R. A. 1991. Filamentous fungi in foods and feeds: ecology, spoilage, and mycotoxin production. In: "Handbook of Applied Mycology, Vol 3, Foods and Feeds," (ed. by Arora, D. K., Mukerji, K. G. and Marth, E. H.), pp 31–68. Marcel Dekker, New York.

Golinski, P., Vesonder, R. F., Latus-Zietkiewicz, D. and Perkowski, J. 1988. Formation of fusarenon X, nivalenol, zearalenone, α-trans-zearalenol, β-trans-zearalenol, and fusarin C by *Fusarium crookwellense*. Appl. Environ. Microbiol. **54**: 2147–2148.

Ichinoe, M., Hagiwara, H. and Kurata, H. 1983. Distribution of trichothecene-producing fungi in barley and wheat fields in Japan. In: "Developments in Food Science Vol 7, Toxigenic Fungi-Their Toxins and Health Hazard," (ed. by Kurata, H. and Ueno, Y.), pp. 190–198. Elsevier, Amsterdam.

Koizumi, S., Kato, H., Yoshino, R., Hayashi, N. and Ichinoe, M. 1991. Distribution of causal fusaria of wheat and barley scab in Japan. Ann. Phytopath. Soc. Japan **57**: 165–173.

Lauren, D. R., Sayer, S. T. and Menna, M. E. 1992. Trichothecene production by *Fusarium* species isolated from grain and pasture throughout New Zealand. Mycopathologia **120**: 167–176.

Miller, J. D., Greenhalgh, R., Wang, Y.-Z. and Lu, M. 1991. Trichothecene chemotypes of three *Fusarium* species. Mycologia **83**: 121–130.

Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. 1983. "*Fusarium* species, an illustrated manual for identification," The Pennsylvania State University Press, PA. 193p.

Nirenberg, H. I. 1990. Recent advances in the taxonomy of *Fusarium*. Stud. Mycol. **32**: 91–101.

Sugiura, Y., Fukasaku, K., Tanaka, T., Matsui, Y. and Ueno, Y. 1993. *Fusarium poae* and *Fusarium crookwellense*, fungi responsible for the natural occurrence of nivalenol in Hokkaido. Appl. Environ. Microbiol. **59**: 3334–3338.

Tanaka, T., Hasegawa, A., Matsuki, Y., Ishii, K. and Ueno, Y. 1985a. Improved methodology for the simultaneous detection of the trichothecene mycotoxins deoxynivalenol and nivalenol in cereals. Food Additi. Contam. **2**: 125–137.

Tanaka, T., Hasegawa, A., Matsuki, Y., Matsui, Y., Lee, U-S. and Ueno, Y. 1985b. Co-contamination of the *Fusarium* mycotoxins, nivalenol, deoxynivalenol, and zearalenone, in scabby wheat grains harvested in Hokkaido, Japan. J. Food Hyg. Soc. Japan **26**: 519–522.

Tanaka, T., Hasegawa, A., Yamamoto, S., Toyazaki, M., Matsuki, Y., Sugiura, Y. and Ueno, Y. 1987. Production of mycotoxins by *Fusarium* isolates from scabby wheat harvested in Hokkaido, Japan. Proc. Jpn. Assoc. Mycotoxicol. **25**: 31–33.

Tanaka, T. and Ueno, Y. 1989. Worldwide occurrence of *Fusarium* mycotoxins, nivalenol, deoxynivalenol and zearalenone. In: "Mycotoxins and Phycotoxins '88," (ed. by Natori, S., Hashimoto, K. and Ueno, Y.), pp. 91–97. Elsevier, Amsterdam.

Thrane, U. 1990. Grouping *Fusarium* section Discolor isolates by statistical analysis of quantitative high performance liquid chromatographic data on secondary metabolite production. J. Microbiol. Methods **12**: 23–39.

Ueno, Y. 1986. Trichothecenes as environmental toxicants. Rev. Environ. Toxicol. **2**: 303–341.

Ueno, Y. 1987. Trichothecenes in food. In: "Mycotoxins in Food," (ed. by Krogh, P.), pp. 123–147. Academic Press, London.