Thirty-five Years of Research on Deoxynivalenol, a Trichothecene Mycotoxin: with Special Reference to Its Discovery and Co-occurrence with Nivalenol in Japan

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Deoxynivalenol (DON), a trichothecene mycotoxin, was characterized together with nivalenol (NIV) from naturally infected wheat and barley grains of the 1970 epidemic in Kagawa**, Japan. DON, 3-acetyl-DON (3-ADON) and 3,15-diacetyl-DON (3,15-DADON) were identified as metabolites of Fusarium roseum No.117 (=F. graminearum ATCC 28114), a toxic isolate from the cereals of the 1970 epidemic, and their toxicological properties in animals, including acute toxicity, emetic activity, and in vivo de-epoxydation metabolism into DOM-1 (deepoxy-DON), were elucidated. Natural co-occurrence of DON and NIV in the domestic cereals was found to be common not only in southern Japan but also in other regions, and the geographic difference in the occurrence of both toxins in Japan was elucidated. In terms of mycotoxigenicity, F. graminearum strains isolated from crop fields were divided into two types: DON- and NIV-producers, and DON-producer was further subdivided into two subtypes, 3-ADON- and 15-acetyl-DON-producers by biotransformation experiment of 3,15-DADON as a precursor. The geographic differences in the incidence of these producers in Japan were observed. Correlation between the incidence of the toxin producers and the occurrence of DON and NIV were investigated by field trials. For screening NIV alone or in combination with DON in cereals, specific monoclonal antibodies were produced, and practical ELISA kits were successfully developed. In relation to our previous findings, current advances in the molecular phylogenetic analysis of mycotoxigenic F. graminearum, the molecular genetics of trichothecene biosynthesis, among others, were briefly reviewed.

Key words: deoxynivalenol, nivalenol, trichothecene, mycotoxin, Fusarium

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**Note: Japan consists of four main islands: Hokkaido, Honshu, Shikoku and Kyushu. Honshu is divided into six regions (districts): Tohoku, Kanto, Hokuriku, Chubu, Kinki and Chugoku. Tohoku is divided into six prefectures including Akita. Kanto is divided into seven prefectures. The other regions in Honshu are divided into 24 prefectures including Shizuoka and Kyoto. Shikoku is divided into four prefectures including Kagawa, and Ehime. Kyushu is divided into seven prefectures including Kumamoto, Fukuoka, and Oita.

Abbreviations: DON, deoxynivalenol; 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; 3,15-DADON, 3,15-diacetyldeoxynivalenol; NIV, nivalenol; 4-ANIV, 4-acetylnivalenol; 4,15-DANIV, 4,15-diacetylnivalenol; 3,4,15-TANIV, 3,4,15-triacetylnivalenol; DOM-1, Deepoxy-deoxynivalenol; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GC-ECD, gas chromatography with electron-capture detection; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; IR, infrared spectrometry; UV, ultraviolet absorbance; MS, mass spectrometry; PMR, proton magnetic resonance spectroscopy; MAB, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.
1. Historical Background

The damage caused by phytopathogenic *Fusarium* species in wheat, barley, and related cereal crops is called *akakabi-hyo* (red-mold disease, scab disease, *Fusarium* head blight) in Japan. The epiphytotic is usually due to wet weather during earing, flowering (anthesis), maturing, and harvest seasons, all of which provide favorable environmental conditions for a severe epidemic in crop fields. The symptoms of diseased kernels involve the development of reddish coloration, shriveling, and a prolonged immature state, imposing a threat to the food supply. For example, major outbreaks of a *Fusarium* invasion in southern Japan occurred in the 1890, 1901, 1914, 1932, 1946, 1958, 1963 and 1970 crops, and yield losses of wheat and barley were remarkable\(^1\). In addition, several intoxications of human and farm animals considered to be associated with the consumption of damaged cereals were reported\(^1\). Two case reports of human intoxication occurred in Hokkaido\(^1\) in 1956 were recorded as *Fusarium*-associated food poisoning\(^1,2\).

Experimental *Fusarium* toxicosis of guinea pigs with oats and wheat cultures of *Gibberella saubinetti* (=*Gibberella zeae*) was reported in 1933\(^3\). A toxic strain of *Fusarium* species to mice was isolated from wheat and wheat flour responsible for human intoxication in 1946\(^4\). In extensive work by Nishikado\(^5,6\), the main causative pathogenic fungus was identified as *G. zeae* (Schw.) Petch (the asexual or conidial stage is *F. graminearum* Schwabe), and found with a maximum contamination rate of 69% in infected barley grains associated with the calf intoxication in 1953. In the 1955 epidemic, unpollished rice grain was damaged 45–83% by *Fusarium* species, as was 15–36% in polished rice. When the naturally infected rice mixed with 10% fish meal was fed to rats for 24 weeks, three of seven rats died within the last 3 weeks after feeding, and loss of body weight was observed in all surviving animals\(^7\). The rice culture of a toxic *Fusarium* isolate was defatted and then extracted with 30% ethanol in water to afford a crude extract, which revealed lethal toxicity to rats at doses of over 9 mg per 10 g of body weight. Lethal toxicity was also shown in a liquid culture of Czapek-Dox medium enriched with 0.5% of peptone. These works served as forerunners for the subsequent mycotoxicological researches on *F. nivale*, *F. graminearum* (*F. roseum*) and related fungi in Japan.

In 1963, a long rainy season in southern Japan resulted in severe damage by red-mold disease in the wheat and barley crops of this region. The Agricultural, Forestry and Fisheries Research Council conducted investigations on the tolerance of various kinds of farm animals to the damaged cereals contaminated with toxic *Fusarium* species, on the permissible level of these cereals mixed in animal feeds, and on the properties of toxic substances involved. Three toxigenic strains, *F. nivale* (Fn-2) and *F. graminearum* (Noken No. 36 and Ohita No. 7) were isolated from the damaged wheat sample\(^8\). Strain *F. nivale* (Fn-2) was isolated from damaged wheat from Kumamoto\(^9\), and its fungal identification was revised later as *F. tricinctum* var., then as *F. graminearum* var., and now as *F. kyushuense* sp. nov.\(^9\). Remarkable cytotoxicity of the crude toxin fractionated from rice culture of *F. nivale* (Fn-2) was demonstrated in subcutaneously injected mice. Cytotoxicity was also confirmed not only by animal experiments revealing the radiomimetic nature of toxins, but also by simple screening tests using animal cell cultures\(^10\). In addition, a biochemical assay method of toxic principles using rabbit reticulocytes was developed\(^11\). These assay systems were successfully applied for isolation of toxic principles from the crude toxin. Thus, nivalenol (NIV) and fusarenon (4-ANIV), new trichothecene metabolites, were isolated from rice cultures of Fn-2 strain\(^12,14\); in addition to these toxic metabolites, fusarenone-X (identical to 4-ANIV) and 4,15-diacetylvalenol (4,15-DANIV) were isolated from culture of Fn-2 grown in 1% peptone-enriched Czapek-Dox medium\(^15,17\). Physicochemical and toxicological properties of these mycotoxins of *F. nivale* (Fn-2) were reviewed\(^18,19\).

2. Characterization of Toxic Substances (DON and NIV) in Naturally Infected Cereals

Until 1970, no attempt was made to characterize the toxicants involved in the *Fusarium*-damaged cereals of field crops. Regarding the characterization of mycotoxins occurring in agricultural products, a typical approach chosen in the majority of previous studies seemed to be as follows: i) surveillance of infected fungi (mycoflora) in certain samples, ii) screening toxigenic fungal isolates, and production of toxic principles metabolized by selected fungal isolates, usually the most toxic ones, in laboratory cultures, iii) isolation and characterization of toxic principles from cultures, and toxicological studies on the toxic principles, and iv) based on outcomes gained in these studies, the occurrence of certain principles in samples might be speculated upon. However, the presence of the fungi in or on a food product does not automatically mean the presence of the associate toxin(s) but the possibility of toxin contamination as many factors are involved in its formation. Conversely, the absence of any visible fungi does not guarantee that the commodity is free of toxins, as mycotoxins may persist long in food chain after the fungi have lost viability. In addition, it is likely that fungi
may produce different toxins in laboratory cultures from those produced in field crops, because nutrient supply and the ecology of fungi under laboratory conditions are quite different from those found under field conditions. Furthermore, naturally occurring toxins could be modified under field conditions by the action of micro-organisms and / or living plants, and by other factors. Thus, studies on the toxic principles in naturally infected cereals are a prerequisite to elucidate directly the actual situation of mycotoxin contamination in samples of concern. Laboratory of Food Hygiene (Prof. N. Morooka), Faculty of Agriculture, Kagawa University, started comprehensive mycological and chemical studies on toxic substances in Fusarium-damaged wheat and barley of the 1970 epidemic in Kagawa.

**2.1 Isolation of Toxic Substances from Fusarium-damaged Wheat and Barley of the 1970 Epidemic**

Three samples of Fusarium-damaged barley grains were collected from the Kanonji district (two samples, 92% and 83% of damaged kernels) and the Miki district (38% of damaged kernels) in Kagawa. Individual grain samples were powdered, defatted with n-hexane, and then suspended in cold water followed by addition of an equal amount of ethanol. After blending, the aqueous alcohol extract was concentrated to dryness, re-dissolved in water, and filtered off insoluble matters. The resultant aqueous solution was treated with activated charcoal followed by elution of absorbed materials with methanol. Most toxicity in the original barley were found in this eluate. Chromatography of the crude extracts on silica gel column using chloroform-methanol (97:3, then 5:1) gave two toxic fractions (fractions d and f) revealing intraperitoneal lethality at dosage of over 1 mg/mouse. Three barley samples collected from two different locations in Kagawa gave two toxic fractions of similar profiles after chromatographic elution. Each fraction was further purified by column chromatography and preparative thin-layer chromatography (TLC) on silica gel, and two toxins, designated as “toxin d” and “toxin f,” were successfully isolated. Each toxin showed a characteristic color reaction (pink to reddish violet) on TLC plate similar to known trichothecenes such as nivalenol (NIV) and 4-acetylnivalenol (4-ANIV) which have a carbonyl group at the C-8 position, when the plate was sprayed with sulfuric acid and heated. This reaction was a key indication for tracing toxicants in the extracts. Yields of both toxins were estimated about 5–7 mg/kg of barley grains. This figure was substantially low because of unavoidable losses during extraction and isolation procedures.

**2.2 Deoxynivalenol (DON)**

Toxin “d”, tentatively named Rd-toxin and later found to be 4-deoxynivalenol (abbreviated DON), formed fine needles when crystallized from ethyl acetate or ethyl acetate-petroleum ether, mp 151–153 °C, $[\alpha]_D^{25} +6.35$ ($c = 0.07$, ethanol). Anal. Found: C, 60.84; H, 6.81%; MS $m/z$: 296.1276 (molecular ion). Calcd. for $C_{15}H_{20}O_7$: C, 60.80; H, 6.80%; 296.1295. UV $[\lambda_{max}]_{EtOH}$ nm (e): 218 (4500). IR $[\nu_{max}]_{KBr}$ cm$^{-1}$: 1680. IR spectrum of Rd-toxin was shown together with MS spectrum in the paper published in 1972. PMR $[\delta_{TMS}]_{CDCl_3}$: 1.15 (3H, s, CH$_3$-C-), 1.92 (3H, d, J = 1.5 Hz, CH$_2$-C-), 3.14 and 3.25 (each 1H, d, J = 4 Hz, CH$_2$-C-), 3.94 (1H, d, J = 4 Hz, CH$_3$-C-), 4.95 (1H, d, J = 6 Hz) and 6.79 (1H, dd, J = 6 Hz, 1.5 Hz, CH=–C-). The presence of ethylene oxide ring was proved from positive epoxide test with sodium thiosulfate and a typical AB system of PMR at $\delta$ 3.14 and 3.25. The presence of $\alpha,\beta$-unsaturated carbonyl group was confirmed by the formation of semicarbazone derivative: mp 199–201 °C (decomposition); UV $[\lambda_{max}]_{EtOH}$ nm (e): 268 $^{18,20}$. The positive Tollens’ test and tetrazolium test suggested the presence of hydroxyl group at $\alpha$-position of a carbonyl group. Acetylation of Rd-toxin with acetic anhydride in pyridine afforded DON triacetate (3,7,15-triacetyldeoxynivalenol, 3,7,15-TADON): mp 155–157 °C (colorless needles from ethyl acetate-petroleum ether). PMR showed singlet resonances due to three methyl protons of acetyl groups were observed at $\delta$ 3.66, 3.98, 4.63, and 4.95 to $\delta$ 4.30, 4.44, 5.26, and 6.08, respectively. These results indicated that three hydroxyl groups were located at the C-15, C-3, and C-7. The latter two hydroxyl groups were suggested in $\alpha$-configuration by comparing the PMR spectrum with that of NIV. Thus, $3a,7a,15$-trihydroxy-1,12,13-epoxytrichothec-9-en-8-one was proposed for DON, newly found in naturally Fusarium-damaged cereals (Fig. 1). DON is a trichotheceene lacking a hydroxyl function at the C-4 position, which is present in almost all known trichotheccene mycotoxins.

**2.3 Nivalenol (NIV)**

Toxin “f” forms needles when recrystallized from methanol, mp 224–226 °C. UV $[\lambda_{max}]_{EtOH}$ nm (e): 219. Anal. Found: C, 57.72; H, 6.45%. Calcd. for $C_{13}H_{20}O_7$: C, 57.68; H, 6.46%. It gave a single spot on silica gel TLC at the same $R_f$ value as NIV. The infrared spectrum was identical with that of NIV. Based on these evidences, toxin “f” was characterized as NIV ($3a,4\beta,7a,15$-tetrahydroxy-1,12,13-epoxytrichothec-9-en-8-one). Thus, the natural contamination of NIV
was confirmed at the first time in naturally infected barley samples\textsuperscript{21)}, although NIV had been previously isolated from a pure culture of F. nivale Fn2B\textsuperscript{13}). In addition, it was noticed that the co-occurrence of DON and NIV was found for the first time in naturally infected cereal grains (Fig. 1).

2.4 The Trivial Name for Deoxynivalenol

The chemical structure of DON was shown to be identical with a swine emetic and refusal principle in a naturally infected com sample by Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois\textsuperscript{23}). During 1972, because of unusually wet weather, much corn produced in some parts of the U.S. Corn Belt was infected in the field by \textit{F. graminearum} Schw., causing feed refusal and vomiting in swine after eating small quantities. An emetic principle was isolated from the infected corn sample from a North Ohio farm. A tentative structure was proposed for the vomiting factor based on mass spectrum, infrared and nuclear magnetic resonance data, and the trivial name of vomitoxin was given for this substance. Although the paper described the presence of a hydroxyl group at the C-7 position, the location and configuration of the other two hydroxyl groups were not determined.\textsuperscript{23)}

After the discovery of DON, two different names, deoxynivalenol (DON) and vomitoxin, had often appeared in publications. In 1997, Dr. L. Stoloff, U.S. Food and Drug Administration, proposed the following recommendation to solve this confusion\textsuperscript{24}). “In the chemistry science community, the privilege of providing a trivial name for a newly characterized compound goes to the person or team that publishes the first formal presentation of its structure. In the case of the emetic factor produced by various species of \textit{Fusarium}, particularly \textit{F. roseum} (=\textit{F. graminearum}), that privilege belongs to the Japanese team headed by T. Yoshizawa (T. Yoshizawa, Agric. Biol. Chem. 37, 2933, 1973) who named the compound “deoxynivalenol”, and not the Vesonder team (R. F. Vesonder, Appl. Microbiol. 26, 1008, 1973) that named the compound ”vomitoxin.” The Japanese team was also the first to present its findings in a formal lecture at an open meeting. It behooves the mycotoxin community to honor the chemists’ convention and use the term “deoxynivalenol” for the \textit{Fusarium} emetic factor. The term “vomitoxin” can be added parenthetically.”

3. Characterization of DON and Its Derivatives Produced by \textit{Fusarium} Species

3.1 Isolation of Toxic Strains of \textit{Fusarium} Species from Wheat and Barley of the 1970 Epidemic

Several fungal genera such as \textit{Fusarium}, \textit{Alternaria}, \textit{Aspergillus} and \textit{Penicillium} were detected in the barley and wheat grains infected during the 1970 epidemic in Kagawa\textsuperscript{25}. Detection rates of \textit{Fusarium} species were clearly correlated with visual damage (mycelia spread) of grains, and the major fungi causing the damage was found to be \textit{F. roseum} or related species. Fungal identification was made according to the Snyder and Hansen’s classification system. Twenty-one strains of \textit{Fusarium} species were isolated from barley and wheat grains of six different locations. Each isolate was surface cultured on 0.5% peptone-supplemented Czapek-Dox media for three weeks, and the culture filtrate was treated with active charcoal followed by elution with methanol to obtain the crude toxic extract. Aliquots of the crude extract
were injected intraperitoneally to DDY mouse for the lethal toxicity and also exposed to protozoan (*Tetrahymena pyriformis*) for the growth inhibition. Of 21 isolates examined, three strains were toxic to either mouse or protozoan, only one strain, *F. roseum* No.117 exhibited toxicities in both tests\(^{21}\). This strain was classified as *F. graminearum* according to the manual of Booth, and registered later as *F. graminearum* ATCC 28114.

### 3.2 Characterization of Trichotheccenes of *Fusarium roseum* (*F. graminearum*)

#### DON and 3-Acetyldeoxynivalenol (3-ADON)

The toxic *F. roseum* No. 117 strain was surface cultured on 0.5% peptone-enriched Czapek-Dox medium at 20 °C for 3 weeks, followed by charcoal treatment of the culture filtrate and methanol elution as described above. The crude extract was fractionated by column-chromatography on silica gel using chloroform-methanol (97:3, then 5:1). The eluate was developed on TLC plate followed by monitoring under shortwave (250 nm) ultraviolet light and by treating the plate with sulfuric acid spray, fractions showing similar patterns were combined, and afforded to toxicity tests in mouse and protozoan. Toxic fractions to both mouse and protozoan were combined and further purified on silica gel column by eluting with ethyl acetate-toluene (3:1). Of three substances purified, two substances were identical to butenolide and Rd-toxin (deoxynivalenol), the other, tentatively named Rc-toxin, showed a similar color reaction (pink to reddish violet) to DON on TLC plate when the plate was sprayed with sulfuric acid followed by heating\(^{20,21}\). Rc-toxin (deoxynivalenol monoacetate) was recrystallized from diethylether-n-pentane, mp 185.5–186 °C. \([\alpha]_D^{25} +40.5^\circ (c = 0.06, \text{ethanol})\). Anal. Found: C, 60.88; H, 6.64%; MS \(m/z\) 338.1388 (molecular ion). Calcd. for C\(_{17}\)H\(_{22}\)O\(_7\): C, 60.55; H, 6.47%. IR \(\nu_{max}\) KBr cm\(^{-1}\): 3480, 3400, 1740, 1680. PMR \(\delta_{TMSi}\) CDCl\(_3\): 1.16 (3H, s), 1.90 (3H, d, \(J = 1\) Hz), 2.14 (3H, s, acetyl group), 3.10 and 3.18 (each 1H, d, \(J = 4\) Hz), 3.76 and 3.91 (each 1H, d, \(J = 12\) Hz), 3.90 (1H, d, \(J = 4.5\) Hz), 4.70 (1H, d, \(J = 5.5\) Hz), 4.84 (1H, s), and 5.24 (1H, m). Chemical and spectroscopic properties of the hydrolysis and acetylation products were identical with DON and 3,7,15-TADON, respectively. Acetylation of the monoacetate was resulted in upward shifts of C-7 proton at \(\delta = 4.84\) and C-15 protons at \(\delta = 3.76\) and 3.98, while hydrolysis resulted in a shift downward of the C-3 proton to \(\delta = 5.24\), thereby indicating a monoacetylated form of the C-3 hydroxyl group of DON. The partial acetylation of DON with acetic anhydride in pyridine gave Rc-toxin in addition to 15-acetyldeoxynivalenol (15-ADON). Thus, 3-ADON was characterized as 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothecc-9-en-8-one\(^{22}\) (Fig. 2).

#### 3,15-Diacetyldeoxynivalenol (3,15-DADON)

*F. roseum* No. 117 strain (=*F. graminearum* ATCC 28114) was cultured on moistened rice grains at 25 °C for 10 to 12 days. Moldy rice grains were blended with 50% aqueous methanol, and the mixture was filtered to obtain the aqueous methanol extract, which were concentrated to dryness and extracted successively with n-hexane and chloroform. The
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chloroform extract was chromatographed on a silica gel column using chloroform-methanol (97:3). In addition to DON and 3-ADON (about 100 and 400 mg/kg of rice culture, respectively), a new metabolite, diacetyldeoxynivalenol, was isolated; the yield of this substance was about 10 mg/kg of rice culture. The metabolite was recrystallized from ethanol, mp 119–120 °C. Anal. Found: C, 59.70; H, 6.30%. MS m/z: 380 (molecular ion). Calcd. for C_{19}H_{24}O_8: C, 59.99; H, 6.34%.

PMR [δ(TMS)CDCl_3]: 1.12 (3H, s), 1.92 (3H, d, J = 1.5 Hz), 1.92 and 2.16 (each 3H, s, two acetyl groups), 3.10 and 3.16 (2H, d, J = 4.5 Hz), 3.77 (1H, d, J = 2 Hz), 3.90 (1H, d, J = 4.5 Hz), 4.20 (2H, s), 4.70 (1H, d, J = 6 Hz), 4.81 (1H, d, J = 2 Hz), 5.22 (1H, m), 6.58 (1H, dd, J = 1.5 Hz, 6 Hz). The PMR data, as compared with that of 3,7,15-TADON, indicated that two acetoxy groups of the compound were located at the C-3 and C-15 positions. The metabolite showed spectroscopic identity with a product obtained by partial acetylation of 3-ADON with an equimolar of acetic anhydride in pyridine. Thus, diacetyldeoxynivalenol was confirmed as 3,15-DADON (3a,15-diacetoxy-7α-hydroxy-12,13-epoxytrichothec-9-en-8-one) (Fig. 2).

4. Toxicological Characterization of DON and Its Related Toxins

4.1 Acute Toxicity

The LD_{50} values, mg/kg of body weight (bw), of DON, 3-ADON and 3,15-DADON were 70, 49 and 145 respectively, when injected intraperitoneally to male DDY mice. The oral LD_{50} values in male mice were 46 mg/kg bw for DON and 34 mg/kg bw for 3-ADON, indicating slightly higher oral toxicities for these toxins than those obtained by intraperitoneal administration. No sexual difference in their toxicities was observed. The acute symptoms of intoxicated mice were marked dilatation with hemorrhage of the gastrointestinal tract and congestion of the testes. The LD_{50} values (mg/kg bw) of NIV, 4-ANIV and 4,15-DANIV were reported 4.1, 3.3 and 9.6, respectively in DDY mice administered intraperitoneally. The relative LD_{50} values calculated for DON, 3-ADON and 3,15-DADON is 1.0: 0.7: 2.1, which is closely comparable to the ratio of 1.0: 0.8: 2.3 shown in NIV, 4-ANIV and 4,15-DANIV, respectively. The lower toxicity of DON to animals as compared with other trichothecenes such as NIV may imply that the C-4 hydroxyl group of trichothecene nucleus plays an important role in mammalian toxicities. On the other hand, compared to 3-ADON, DON showed higher cytotoxicity to protozoan, HeLa cell, chick embryofibroblast cell, and sea urchin egg. This indicates that the deacetylation of 3-ADON results in an increase in selective toxicity.

4.2 Feed Refusal and Emetic Activity of DON

Moldy cereals infected with Fusarium or Gibberella species are known to cause intoxications such as emesis, diarrhea, and diet refusal in man and animals. The subcutaneous minimum dose of DON for emesis and diarrhea was 0.1 mg/kg bw in 6-month-old dog, and no symptom was observed at 0.025 mg/kg bw. The first and final vomiting were observed around 15 and 55 min, respectively, after administration with 0.1–0.2 mg DON/kg bw, and around 9 min and 210 min, respectively, with 12 times of vomiting after administration with 1.0 mg DON/kg bw. As for 3-ADON, the minimum dose for emesis and diarrhea was estimated 0.2 mg/kg bw with only one vomiting at around 60 min, and the first and final vomiting at a dose of 1.0 mg/kg were at 17 min and around 170 min, respectively, with five bouts of vomiting. Thus, in contrast with the acute toxicity, the emetic activity of DON in dogs is apparently higher than that of 3-ADON.

Furthermore, the emetic and refusal activity of purified DON to swine was studied in corporation with Purdue University, Indiana, USA. The minimum emetic dose of DON given intraperitoneally or orally to swine weighing 9 to 10 kg was 0.05 mg/kg bw or 0.1 to 0.2 mg/kg bw, respectively. No emesis was observed in pigs fed vomitus from pigs orally dosed with DON or penned with such pigs without access to vomitus. DON added to feed reduced feed consumption of 20- to 45-kg pigs, ranging from a 20% decrease with 3.6 mg DON/kg feed to 90% reduction with 40 mg DON/kg feed. The feed refusal, however, was much greater for the naturally Gibberella zeae-infected corn samples than for feeds with equal concentrations of the pure DON added. This finding indicated that the G. zeae-infected corn contained other active compounds than DON, although DON is an important emetic and refusal compound. Later, 15-acetyldeoxynivalenol (15-ADON), DON and zearalenone were found in corn refused by swine at concentrations of 16 mg/kg, 20 mg/kg and 5 mg/kg, respectively.
4.3 De-epoxydation of DON – Detoxification in Animals

In the course of studies on the in vivo metabolic fate of DON in animals, the author found that the 12, 13-epoxy-ring of DON molecule was reduced to form a de-epoxy metabolite\(^{30}\). After oral administration of DON to Wistar rats, an unknown metabolite designated as DOM-1 was found in the rat urine and feces. Urinary and fecal elimination completed by 72 hr, 4.5% and 4.4% of the administered dose being recovered from urine for DON and DOM-1, respectively, and 0.3% and 5.6% of the administered dose being recovered from feces for DON and DOM-1, respectively. DON was detected also in the plasma and liver at 12 hr after dosing at concentrations of 43 and 63 ng/g, respectively, and DOM-1 was at concentrations of 24 and 16 ng/g, respectively\(^{30}\).

By GC-MS of the trimethylsilyl (TMS) ether derivative of DOM-1, its molecular ion was found at m/z 496.2477 (calcd for C\(_{24}\)H\(_{44}\)O\(_5\)Si\(_3\), M.W. 496.2493), suggesting the elimination of one oxygen atom and the retention of three hydroxyl groups at the C-3, C-7 and C-15 positions. Other fragments at m/z 481 (M-15), 406 (M-90), 391 (M-105), 376, 361 and 309 were also shifted by 16 mass units (one oxygen atom) as compared with corresponding ions in the TMS ether of DON. The metabolite was negative to the color reaction on TLC plate after treating with 4-([p-nitrobenzyl]-pyridine, suggesting the absence of the 12, 13-epoxy function. By PMR of DOM-1, instead of a singlet resonance at δ 3.03 due to methylene protons of the epoxy ring in DON, doublet resonances were observed at δ 4.92 and 5.09 (each 1H, J = 0.99 Hz) in DOM-1, which were assigned to terminal methylene protons at the C-13 position. According to the formation of this double bond, a singlet signal (δ, 1.09) of methyl protons at the C-14 in DON was shifted to the low magnetic field, whereas a methine proton at δ 4.81 (C-7) was shifted to δ 4.58 due to magnetic anisotropy. By CMR of DOM-1, signals at δ 66.42 (C-12, s) and 47.50 (C-13, t) in DON were shifted to δ 155.39 and 106.38, respectively. Furthermore, by treating with m-chloroperbenzoic acid in chloroform, the metabolite was quantitatively converted into DON. Based upon these data, 3α, 7α, 15-trihydroxytrichothec-9,12-dien-8-one (Fig. 3) was proposed for the chemical structure of the newly found metabolite, DOM-1\(^{30}\). In view of the metabolism of xenobiotics, it is worth noting the direct deoxygenation (de-epoxydation) at an epoxide ring to form a double bond in animals.

This metabolic reaction was also found to be common in other trichothecenes such as T-2 toxin, diacetoxyscirpenol, and NIV\(^{31–33}\), and intestinal microflora of rat was found to be associated with the reaction\(^{34}\). Later, successful trichothecene transformation experiments were conducted by many researchers with ruminal or gut microflora, indicating that mixed cultures of anaerobic microorganisms can detoxify DON by enzymatic reduction\(^{35–42}\). Nevertheless, no pure culture of associate microbes could be isolated until 1997, when a novel strain of *Eubacterium* sp. (*Eubacterium* BBSH797) with the ability to transform DON to DOM-1 was isolated\(^{43}\). Nowadays, the encapsulated bacterium is included in a commercial formulation designed for poultry and swine diets (http://www.biomin.net/at/home).

Recently, conventional microbial selection strategies guided by PCR-DGGE (denaturing gradient gel electrophoresis) bacterial profiles were used for isolating DON-transforming bacteria\(^{44}\). This approach has significantly increased the efficiency of bacterial selection, and ten isolates from chicken intestines, most potent in transforming DON to DOM-1 during sub-culturing, were shown to belong to four different groups: *Clostridiales*, *Anaerofilum*, *Collinsella*, and *Bacillus*. Microbial detoxification is considered to be the only effective way to detoxify mycotoxins that can be only poorly
bound by adsorbents, namely ochratoxin A, zearalenone as well as DON. The new yeast strain, *Trichosporon mycoxin-vorans*, isolated from a hindgut of lower termites was found suitable to detoxify both ochratoxin A and zearalenone\(^{45}\).

5. Natural Occurrence of DON and NIV in Japan

The discovery of DON and its co-occurrence with NIV in the 1970 epidemic cereals led us to proceed to the next phase, and the following questions had risen to be clarified: i) whether the natural occurrence of DON/NIV is sporadic and accidental in an epidemic year in a limited area, ii) the possibility of incidence of both DON/NIV in a normal harvest year, iii) the diversity of the fungal origin of DON/NIV, and iv) the distribution of DON/NIV contamination and *Fusarium* species producing DON/NIV. To promote these investigations, the development of sensitive and selective method for detection of these toxins was prerequisite. Regarding the determination of trichothecenes occurring in foods and feeds, the following steps are included in the analytical procedures: solvent extraction of toxins from samples, column cleanup of extracts, derivatization of toxins in some cases, and separation and quantitation of toxins\(^{46}\). In the low 1980s, aqueous acetonitrile or methanol was chosen for solvent extraction of trichothecenes from cereal samples\(^{47,48}\) and Amberlite XAD-2\(^{49}\), silica gel\(^{48}\), Florisil\(^{47}\), charcoal-alumina-Celite column\(^{50}\) or solid-phase extraction column\(^{51}\) was used for cleanup procedure. Finally, trichothecenes were determined by GC with electron-capture detection\(^{52,53}\) and GC-MS\(^{47}\) as various derivatives, or by HPLC\(^{51}\).

5.1 Co-occurrence of DON and NIV in Wheat and Barley Grains

**Co-occurrence in Shikoku and Kyushu, Southern Japan**

In 1976 to 1982, 205 samples of wheat and barley grains were collected in Shikoku\(^*\) and Kyushu\(^*\) districts and surveyed the contamination of DON and NIV. Among samples collected during 1976 to 1980, very heavily damaged samples at a level of more than 5 mg/kg were included. These were the grains separated by a winnowing machine before marketing and usually supplied for animal consumption or discarded. As for the chemical analysis of DON and NIV, a ground sample was extracted with water-methanol (1:3) followed by cleanup on Amberlite XAD-2 and Florisil columns according to the published method\(^{49,53}\) with some modification. After derivatizing DON and NIV to trimethylsilyl (TMS) ethers, the quantification was performed by GC-ECD with an OV-17 packed column. The incidence of DON and NIV in wheat and barley grains was as high as 74.6% (153/205 samples). In 61.5% of total samples, approximately equal levels of both mycotoxins co-occurred regardless of crop year, habitat, variety and cultivar of the cereals. The incidence of grains contaminated with DON or NIV at the levels of ≥2 mg/kg, ≥1 mg/kg or ≥0.3 mg/kg was 17.6% (36/205 samples), 27.8% (57/205) or 46.8% (96/205), respectively\(^{54}\).

**Geographic difference in the Co-occurrence of DON and NIV in Japan**

To clarify characteristics in the profile of DON and NIV occurrence in Japan, a total of 245 grain samples of wheat and barley were collected from 88 crop fields in 16 prefectures in 1984 to 1994 and analyzed for these mycotoxins by GC-MS\(^{55,56}\). The mycotoxins were detected in 190 (78%) samples: NIV and DON were in 178 and 153 samples, respectively, and 144 samples were co-contaminated with both toxins. The results indicated the regional difference in the DON and NIV contamination of Japanese wheat and barley (Fig. 4)\(^{55-57}\). Generally speaking, DON was the major toxin in the northern district, Akita\(^*\) and Hokkaido\(^*\) (approximately 70% DON in concentration) and NIV in the central districts (over 80% NIV), whereas in the southern districts the DON level was 30 to 50% in Shikoku\(^*\) district, and was slightly higher (55 to 65%) than the NIV level in Kyushu\(^*\) district. Several data previously obtained in Hokkaido\(^*\), Kanto\(^*\), and Hokuriku\(^*\) districts were in line with those described in this study. Thus, it should be emphasized that these results of the different profile on the occurrence of DON and NIV are highly correlated to the geographic difference in the distribution of different chemotypes of *Fusarium* (Gibberella) species producing these trichothecenes in wheat and barley grains and also in fields of these crops in Japan, as discussed in the following section.

5.2 Occurrence of Acetylated Derivatives of DON and NIV in Japan\(^{62}\)

Thirty-four samples of domestic wheat and barley grains, collected from eight prefectures of different locations, namely Hokkaido\(^*\), Akita\(^*\), Tochigi\(^*\), Kyoto\(^*\), Kagawa\(^*\), Ehime\(^*\), Fukuoka\(^*\), and Oita\(^*\), and previously determined to be positive for DON and NIV, were analyzed for acetylated derivatives of DON and NIV by GC-MS. In addition to DON and NIV, 3-ADON, 15-ADON and 4-ANIV were found in 25, 4 and 14 samples, respectively. A regional difference in
the DON and its acetates contamination of Japanese wheat and barley was suggested, 3-ADON occurred together with DON in almost all prefectures examined, whereas 15-ADON was found only in samples from northern districts (Hokkaido** and Akita**). In addition, a high correlation \( r = 0.974, n = 23 \) between levels of DON and those of its acetates (3-ADON and 15-ADON) was noted, when statistically analyzed on the samples contaminated with any trichothecenes at levels of over 10 µg/kg. In this case, the DON level was always higher than those of its acetates, the DON/(3-ADON plus 15-ADON) ratio ranging from 2.9 to 155. On the other hand, the occurrence of 4-ANIV seemed to be less frequent than that of 3-ADON, with the positive correlation \( r = 0.633, n = 9 \) between levels of NIV and those of 4-ANIV in the samples contaminated at levels of over 10 µg/kg.
Since DON producers of *F. graminearum* can be subclassified into two types: 3-ADON producer and 15-ADON producer (see Section 7.1), the presence of monoacetylated DON in cereal grains would be considered as an indication of the subclassified DON-producers involved in the field infection of cereals. The presence of these acetylated toxins is of importance because they have been found both in feeds associated with field outbreaks of animal mycotoxicoses and in staple foods collected from high-risk areas of human chronic diseases. Furthermore, considering that, in comparison with the estimated acute toxicities (LD₅₀ values) of DON and NIV, those of the monoacetylated derivatives are greater by the same administration route, much attention should be devoted to the natural occurrence of these acetylated trichothecenes together with DON and NIV and the combined effects of these toxins on human and animal health. During discussions for risk assessment of DON and NIV conducted by the Food Safety Commission of Japan, information on the safety of DON and NIV analogues (acetylated, glycosidic and other analogues) was regarded necessary for improvement of the Commission’s risk assessment.

### 5.3 Occurrence of DON and NIV in Marketed Grains and Commercial Foods

Following the above surveillance, the determination of DON and NIV levels in wheat and barley grains, which were purchased from farmers by the government, was attempted. In Japan, the inspection and grading of these cereals are based on the inspection standards including the volumetric weight, uniformity of grains, moisture content and the content of damaged grains. As for *Fusarium*-damaged kernels distinguished optically by their pink coloration, the content of these kernels in the purchased cereals was regulated below 1% for wheat and barley of all grades (Grades 1 to 3, and Off-grade), and below 0.4% for barley grains intended to be used for beer fermentation. Later, this inspection standard was revised in 2003 to set at 0.0% (0.04% for beer fermentation). Forty-nine samples of wheat and barley grains purchased by the government were supplied from Kagawa** Inspection Station for Agricultural Products in 1981 and 1982. The incidence of contaminated samples in individual grade was as follows: Grade 1; 61.5% (8/13), Grade 2; 75% (9/12), Grade 3; 100% (13/13), and Off-grade; 81.8% (9/11). The mean concentration ranged from 107 to 209 µg/kg for DON and from 211 to 483 µg/kg for NIV. Three (6.1%) samples of Grades 1 and 3 were highly contaminated with the level higher than 1 mg/kg of the toxins: DON, 1.12 mg/kg ; NIV, 1.0 and 3.4 mg/kg. Since the majority of these cereal grains of all grades were supplied for processing of various foodstuffs, it is likely that both DON and NIV may have entered the food chain to contaminate several food products to some extent.

To clarify the possibility of DON and NIV contamination in commercial foods, parched-barley flour products, which is called “mugi-kogashi” or “hattaiko,” and produced by roasting whole barley grains followed by flouring, were collected at local markets in Kagawa**, Ehime**, and Kumamoto** Prefectures in 1982. The concentrations of DON and NIV were determined by GC-MS (mass fragmentgraphy). Both mycotoxins were detected in all six samples tested at a level ranging from 27 to 85 µg/kg for DON, and from 37 to 190 µg/kg for NIV. To our knowledge, this is the first report on the natural co-occurrence of DON and NIV in commercial foodstuffs. In 1933, the human vomiting and diarrhea associated with the consumption of parched-barley flours made from *Fusarium*-damaged barley grains was described. A similar poisoning occurred in 1955 in Shizuoka** Prefecture was also recorded. The presence of residual DON and NIV in the food product may suggest that the toxins could be associated with those historical human intoxications.

Further surveillance of cereal products intended for human consumption was carried out in the author’s laboratory during 1996 to 1999. A total of 142 samples of commercially marketed cereal food products were analyzed for DON and NIV contamination using GC-MS. All the samples were produced from domestic wheat and barley. DON and NIV were found in 46 (32%) and 40 (28%) samples, respectively. Yearly averaged levels of DON and NIV ranged from 123 to 513 µg/kg for DON and from 30 to 149 µg/kg for NIV, and maximum levels ranged from 398 to 1,884 µg/kg for DON and from 30 to 717 µg/kg for NIV. It was noteworthy that a considerably high level (1,884 µg/kg) of DON was found in whole wheat flour in 1999 (our unpublished results).

### 5.4 National Monitoring of DON and NIV since 2002

Ten years after the discovery of DON, a number of publications on the toxin increased rapidly year-by-year, and its world-wide occurrence has become well recognized. The evaluation of DON by international organizations has been carried out: International Programme on Chemical Safety (IPCS/WHO) in 1990, International Agency for Research on Cancer (IARC/WHO) in 1993, and JECFA in 2001.

Following the establishment of a temporary regulation limit of DON in unpolished wheat (1.1 mg/kg) in May 2002 by the Ministry of Health, Labour and Welfare (MHLW), the Ministry of Agriculture, Forestry and Fisheries (MAFF) added DON in imported wheat for the inspection program. Ongoing surveillance of mycotoxin contents in domestically
produced wheat and barley have been conducted since fiscal year 2002 with both DON and NIV being included in the test items.\(^{75}\)

The results of the surveillance of DON in domestically produced wheat showed year-to-year variation, with the proportion of samples at or above the quantification limits ranging from 36 to 84% and the mean value from 0.015 to 0.16 mg/kg. None has been confirmed to contain DON beyond the temporary limit, except in fiscal year 2002. As for DON content in domestically produced barley, the proportion of samples at or above the quantification limit ranged from 37 to 100% with the mean value from 0.060 to 0.55 mg/kg. NIV content has been surveyed together with DON. In wheat and barley, the proportion of samples at or above the quantification limit ranged from 32 to 70% and from 56 to 90%, respectively, with the mean value ranging from 0.010 to 0.087 mg/kg and from 0.042 to 0.58 mg/kg, respectively.

These results of the surveillance of DON and NIV are summarized separately for individual mycotoxins. Since the co-occurrence of both toxins is a characteristic feature especially in domestically produced wheat and barley, the estimation of DON and NIV levels in individual cereal samples are recommended.

6. Occurrence of Trichothecene Producing \textit{Fusarium} Species

6.1 Incidence of DON- and NIV-producing \textit{F. graminearum} in Southern Japan\(^{76}\)

Following the identification of DON and NIV in the \textit{Fusarium}-damaged barley of the 1970 epidemic in Kagawa, it had been demonstrated by subsequent studies that both trichothecenes occurred in \textit{Fusarium}-infested cereals of normal harvest years. However, the distribution and incidence of fungi producing these mycotoxins were remained to be elucidated. To clarify this subject, during May to June in 1976, wheat and barley ears were freshly collected from 74 sites of four prefectures in Shikoku and husked immediately after harvest. Wheat and barley kernel samples from 21 sites of seven prefectures in Kyushu were supplied about a month after harvest from Agricultural Experiment Stations of the prefectures. Representative \textit{Fusarium} species isolated were examined for the productivity of several \textit{Fusarium} toxins on both liquid and polished rice media. A total of 106 isolates belonged to the species \textit{F. graminearum}. The incidence of fungal isolates revealing lethal toxicity to mouse and skin necrosis in rats were 22.6% (24/106) and 34.9% (37/106), respectively. The incidence of \textit{Fusarium} toxin producing strains was 39.6% (42/106), 34.9% (37/106) and 16.0% (17/106) for trichothecenes, butenolide and zearalenone, respectively. About half of these toxigenic strains showed the ability to produce two or more mycotoxins simultaneously. Thus toxigenic \textit{Fusarium} strains were detected with a high incidence from pre-harvested and/or freshly harvested barley and wheat grains. Among them, the species \textit{F. graminearum} was predominant and widely distributed in crop fields of southern Japan. Furthermore, the majority of trichothecene-producing strains of \textit{F. graminearum} metabolized either DON/3-ADON or NIV/4-ANIV. These results provided, for the first time, the conclusive evidence for infection of field crops of southern Japan with different strains (i.e. chemotypes: DON-producer and NIV-producer) of \textit{F. graminearum} which can produce either DON or NIV. Our finding on this typing of \textit{F. graminearum} was later confirmed by the subsequent research.\(^{77}\)

6.2 Geographic Distribution of DON- and NIV-producing \textit{G. zeae} (\textit{F. graminearum}) in Japan\(^{55,56}\)

\textit{G. zeae} (417 isolates) were isolated from perithecia on rice-stubbles collected in April to May during 1990 to 1993 from 108 locations in 28 prefectures, and \textit{F. graminearum} (139 isolates) were from 57 kernel samples of wheat and barley collected in 1989 to 1994 from 10 prefectures. Among these 556 isolates, as high as 96% (534 isolates: \textit{G. zeae}, 398 and \textit{F. graminearum},136) of isolates showed mycotoxigenicity on polished rice cultures, and were also divided into NIV- and DON-chemotypes, consisting of 430 (\textit{G. zeae}, 343 and \textit{F. graminearum}, 87) and 104 (\textit{G. zeae}, 55 and \textit{F. graminearum}, 49) isolates, respectively. NIV-producer was predominant (ca. over 90%) in Honshu except central/northern Tohoku and western Chugoku, and about 70 to 80% in the neighboring areas to the above, whereas DON-producer was predominant (ca. over 65%) in northern Tohoku, Hokkaido, and Kyushu except Oita. Similar data had been obtained in Tohoku, Kantou, Hokkaido, and Shikoku (Table 1). As for DON-producers, 3-ADON subtype distributed nationwide while 15-ADON subtype localized in the northern region; Tohoku and Hokkaido. Although NIV-producing \textit{F. graminearum} was not isolated from kernel samples of Akita and Hokkaido in our study, \textit{F. poae} and \textit{F. crookwellense} were reported as NIV-producers in Hokkaido by other researchers.\(^{59}\).
Regarding the mechanism of the epidemic of head blight disease and mycotoxin occurrence in wheat and barley crops, the following sequential steps are involved: i) the formation of *G. zeae* perithecia on plant debris (e.g. overwintered rice-stubbles), ii) aerial dispersal of *G. zeae* ascospores from the perithecia, iii) *G. zeae* infection at anthesis and colonization on spikes under suitable conditions, and iv) *F. graminearum* growth and accumulation of mycotoxins during head maturation.

A 3 year field study (1990–1992) on the incidence of dispersal ascospore of toxigenic *G. zeae* was conducted in a fixed field point with wheat-rice rotation in Kagawa**. The incidence of *G. zeae* ascospores was determined by counting colony formation units (CFU) on the Komada agar plate. Dispersal *G. zeae* ascospore was found in 70 out of 106 trials. The monthly incidence of the dispersal ascospores was the highest (21.9 CFU/plate, all *G. zeae* positive for 12 trials) in April, when wheat enters its spiking and flowering season and is known to be the most susceptible to the infestation of *G. zeae*. The second highest incidence was in July to October (rice-cultivating season, 2.9–4.9 CFU/plate). The lowest incidence (0.2–0.6 CFU/plate) was found in the period from November to February. It should be emphasized that almost all ascospores (172 out of 177 isolates, 97%) exhibited the ability to produce trichothecenes. In terms of their toxigenicity, they were also divided into two types: NIV-chemotype and DON-chemotype, with the incidence of 86% (148 isolates) and 14% (24 isolates), respectively.
In the previous study carried out in author’s laboratory, NIV-producer comprised greater than 95% of the primary inoculum, G. zeae, in wheat and barley fields in Kagawa. However, the co-contamination with NIV and DON is often found in the harvested grains. The frequency of contamination of NIV, DON and their acetylated derivatives in wheat and barley spikes at the early stage of head maturation was studied to understand the mechanism of grain contamination in crop fields. A total of 194 wheat and barley spikes showing head blight symptoms were collected randomly in May 2002 from fields located at Kagawa University Farm, Miki. Of these symptomatic spike samples, 169 spikes (87%) were contaminated with the following trichothecenes: NIV/4-ANIV (132 spikes, 68%), DON/3-ADON (32 spikes, 16%), DON/15-ADON (1 spike), and NIV + DON/3-ADON (4 spikes, 2%). The presence of NIV- and DON-producing strains of F. graminearum was confirmed in a subset of spike samples. Sixteen strains produced NIV/4-ANIV, four produced DON/3-ADON, and only one produced DON/15-ADON in polished rice cultures. These results suggest that the natural contamination of spikes is resulted from an independent generation of DON and NIV by individual toxin-producers, and the spike infection by either NIV- or DON-producer is common, whereas the probability of simultaneous infection by both producers seems to be low in crop fields.

Furthermore, a change in the toxin contamination profile was observed, when damaged spikes were stored at 2 °C and analyzed over a period of 110 days after sampling. Concentrations of the acetylated derivatives gradually decreased to undetectable levels. In NIV-contaminated spikes, the frequencies of 4-ANIV compared to those of NIV were 23% (6 days), 15% (22 days), 1% (62 days) and 0% (104 days). A similar trend was observed in DON-contaminated spikes, wherein the frequencies of 3-ADON were 16% (6 days), 19% (29 days), 3% (62 days) and 1% (107 days). Thus, the conversion of 4-ANIV and 3-ADON to the parent toxins was indicated in the spikes, supporting the author’s previous hypothesis that the deacetylation in the field is induced by biological hydrolysis during the maturation of cereal grain, probably by host-plant enzymes.

### 6.5 Correlation between the Incidence of DON- and NIV-producers in Crop Fields and the Occurrence of DON/NIV in Cereals

To illustrate the mechanism of grain contamination in crop fields, the following five factors were compared: i) the incidence of DON- and NIV-producers of G. zeae/F. graminearum on the inoculums source (perithecia on rice-stubbles), ii) that in aerial dispersal G. zeae ascospores, iii) that on spikes of wheat/barley at maturation, and iv) that in harvested grains, and v) the contamination levels of DON and NIV in harvested grains. As shown in Table 2, the ratio of NIV- to DON-producers was: i) 96:4 in the perithecia on plant debris, ii) 85:15 in the dispersal ascospores, iii) 76:24 on the spikes, and iv) 65:35 in the harvested grains. The ratio of the NIV/DON level in the grains was 60:40 (v).

**Table 2.** Relationships between the incidences of NIV- and DON-producers of G. zeae from perithecia on rice stubbles and from dispersal ascospores, the incidences of those producers of F. graminearum from spikes and grains of wheat and barley, and the contamination levels of NIV/DON in harvested grains

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Perithecia on rice stubbles a)</th>
<th>Aerial dispersal ascospores b)</th>
<th>Wheat/barley spikes at maturation c)</th>
<th>Harvested grains</th>
<th>Toxin level in grains (µg/kg) e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio (%) NIV</td>
<td>No. of G. zeae (ascospore) producing</td>
<td>No. of G. zeae (ascospore) producing</td>
<td>No. of F. graminearum strains producing</td>
<td>No. of spikes contaminated with F. graminearum strains producing d)</td>
<td>13 (65.0%)</td>
</tr>
<tr>
<td>NIV 27 (96.4%)</td>
<td>16 (76.2%)</td>
<td>136 (81.0%)</td>
<td>479 (59.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON 1 (3.6%)</td>
<td>5 (23.8%)</td>
<td>36 (19.0%)</td>
<td>324 (40.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) G. zeae from perithecia on over-wintered rice stubbles collected from eight different fields of wheat and barley in Kagawa b).

b) G. zeae from aerial dispersal ascospores collected in a wheat field in Kagawa, April to May in 1990 and 1991 b).

c) F. graminearum isolated from wheat and barley spikes at maturation in Kagawa University Farm, May in 2002 c).

d) F. graminearum isolated from grain samples harvested from the wheat field in 1991 d).

e) Mean levels of NIV and DON in grain samples harvested from the wheat field in 1990 and 1991 e).
Thus, the profile of NIV- and DON-producers ratio on the inoculum source was clearly reflected in spikes and grains through aerial inoculum dispersal. In addition, compared to the high incidence of NIV-producer in the inoculums source and dispersal ascospores, the incidence of DON-producer in host plants seems to be increased. This is important from the viewpoint of possible differences between DON- and NIV-producers in pathogenic properties such as pathogenicity and aggressiveness, and/or mycotoxin productivity on host plants.

6.6 Current Status of Molecular Genetic Approaches to Mycotoxigenic *F. graminearum* and Their Distribution in Japan

Recent researches on molecular phylogenetic analyses based on eleven nuclear genes at six loci have revealed that *F. graminearum* consists of at least nine biogeographically structured lineages\(^{(87,88)}\). Among nine lineages of the species, now named as *F. graminearum*-complex (=*F. graminearum* Group 2) according to the new concept, *F. graminearum* Schwabe s. str. (lineage 7) and *F. asiaticum* O’Donell *et al.* (lineage 6) are most significant pathogens in wheat, barley and corn in Asia\(^{(89)}\). There is a geographical difference in the distribution of these lineages in Asian countries\(^{(90)}\). For example, *F. asiaticum* is predominant in Chinese wheat followed by *F. graminearum*\(^{(91)}\), whereas in Korea *F. graminearum* is the most predominant followed by *F. boothii* (linkage 3) and *F. asiaticum* in corn and *F. asiaticum* is predominant in rice\(^{(92,93)}\).

From symptomatic wheat and barley heads harvested in 35 prefectures, 298 strains of the *F. graminearum* species complex were collected to determine the species and trichothecene chemotypes. Phylogenetic analyses and species-diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-FRLPs) revealed the presence and differential distribution of *F. graminearum* sensu strict (s. str.) and *F. asiaticum* in Japan\(^{(94)}\). *F. graminearum* s. str. was predominant in northern and northeastern regions, especially in Hokkaido\(^{**}\), while *F. asiaticum* was predominant in central to southern regions. In the Tohoku\(^{**}\) area, these species co-occurred. Regarding trichothecene chemotyping of these lineages by multiplex PCR, all of *F. graminearum* isolates (lineage 7) produced DON, which were subdivided into two chemotypes: 3-ADON producer (about 70%) and 15-ADON producer (about 30%), and none was the NIV-type. *F. asiaticum* isolates (lineage 6) were of either NIV producer (about 70% of isolates) or 3-ADON producer (about 30% of isolates, 15-ADON producer were below 1%)\(^{(95)}\).

Thus, the author’s early findings on the geographical difference in distribution of DON- and NIV-producers in Japan is clearly supported by the current molecular genetic studies on *F. graminearum*-complex producing trichothecene mycotoxins.

7. Limited Studies on Biotransformation of Trichothecenes in *Fusarium* Species

7.1 Regioselective Deacetylation

In laboratory cultures, some DON-producing strains of *F. graminearum* were found to co-produce 3-ADON and DON, whereas the others co-produce 15-ADON and DON. In addition, a trace amount of 3, 15-DADON was also co-produced. Intact mycelia of one type deacetylated 3,15-DADON and 15-ADON at the C-15 position to give 3-ADON and DON, respectively, whereas those of the other type converted 3,15-DADON and 3-ADON into 15-ADON and DON, respectively, by deacetylation at the C-3 position\(^{(96)}\). Similar results were obtained by using cell-free extracts of individual types of *F. graminearum*. Mycelial enzymes involved in these deacetylations were considered to have different properties in terms of optimum pH, pH-stability and substrate specificity. Very low activity of the C-15 deacetylation was shown in all strains examined of non-DON-producer of *F. graminearum*. From these results, it was proposed that DON-producing strains can be divided into two types with respect to the production of DON monoacetate: 3-ADON-producing type and 15-ADON-producing type, and that a precursor, 3,15-DADON, is converted regioselectively by each type of the strain into either 3-ADON or 15-ADON by deacetylating enzymes\(^{(96)}\).

7.2 Acetyl Conjugation of Trichothecenes\(^{(97)}\)

The author first found the microbial acetylation at the C-3α position of DON with resting mycelia of *F. nivale* Fn2B (=*F. kyushuense*)\(^{(86)}\). The acetyl conjugation of T-2 toxin, HT-2 toxin and neosolaniol, each having a C-3α hydroxy group, was also studied by using mycelia of trichothecene-producing strains of *F. graminearum*, *F. nivale* (=*F. kyushuense*), *Calonectria nivalis*, and *F. sporotrichioides*\(^{(97)}\). T-2 toxin was efficiently converted into acetyl T-2 toxin by all
strains except a T-2 toxin-producing strain of *F. sporotrichioides*. HT-2 toxin was conjugated to 3-acetyl HT-2 toxin as an only product by mycelia of *F. graminearum* and *C. nivale*, but was resistant to conjugation by both *F. nivale* and *F. sporotrichioides*. Only *F. graminearum* transformed neosolaniol into 3-acetylneosolaniol. This is the first report on the biological 3α-O-acetyl conjugation of T-2 toxin and its derivatives. The acetyl conjugation of T-2 toxin was also observed in vegetative mycelia of some trichothecene non-producing fungal species (our unpublished results). The biological 3α-O-acetylation of trichothecenes, diaacetoxyscirpenol and 15-acetoxyscirpen-3,4-diol by trichothecene non-producing fungi of *Mucor mucedo* and *F. oxysporum* sp. *vasinfectum* was reported.

From these findings, it was strongly suggested that microbial acetyl conjugation of trichothecene mycotoxins occur predominantly at the C-3α position by trichothecene producing fungi, but not by all, and that microorganisms having this activity will often found in nature.

### 7.3 Microbial Conversion of DON into NIV

Microbial conversion of DON was attempted using *F. graminearum* No.1383, a strain producing NIV, 4-ANIV and 4,15-DANIV. Washed mycelia of vegetative growth of the fungus was incubated in a phosphate buffer containing [14C] DON. The reaction products were analyzed by reversed-phase HPLC radio-chromatography. Eighty two % of the added substrate was efficiently converted into NIV and its acetates (4-ANIV and 4,15-DANIV) within 24 hr. The transformation was also attempted on a solid phase using polished rice spiked [14C] DON. After incubation of *F. graminearum* No.1383 for 24 days, the substrate was completely metabolized to afford [14C]-labeled NIV (24% of the products), 4-ANIV (72%) and 4,15-DANIV (4%). Thus, this microbial hydroxylation at the 4β-position of DON is particularly interesting event from the viewpoints of both the biogenesis of NIV and the evolutional relationship between DON-producer and NIV-producer. Later, the transformation of DON into NIV and its acetates by *Fusarium* sp. producing diaetoxyscirpenol was reported.

### 7.4 Current Studies on Biosynthetic Genes of Trichothecene Mycotoxin in *Fusarium* Species

Molecular genetic approaches to the biosynthesis of trichothecene mycotoxins have been performed extensively in the last 15 years. As for the organization and orientation of genes in *F. graminearum*, seven genes in the cluster (Tri3, Tri4, Tri5, Tri7, Tri8, Tri11 and Tri13) code for enzymes in the trichothecene biosynthetic pathway; two genes (Tri6 and Tri10) code for regulation of other genes; one gene (Tri12) codes for a transporter; two genes (Tri9 and Tri14) are of unknown function. Four genes (Tri101, Tri1, Tri16 and Tri15) that are involved in trichothecene biosynthesis have been found to be located outside the gene cluster.

Comparison of the trichothecene biosynthetic gene cluster between DON and NIV producers revealed that Tri7 and Tri13 were aberrant in the DON producers. It was also shown by genetic engineering that a functional Tri3 coding C-4 monooxygenase converts DON into NIV, and a functional Tri7 coding by 4-O-acetyltransferase converts NIV into 4-ANIV, and Tri11 and Tri14 code monooxygenase at C-8, C-2 and C-15, respectively, and Tri3 codes C-15-acetyltransferase. Tri7, a gene for 3-O-acetylation, was isolated as the first trichothecene resistance gene as an only product by mycelia of *Mucor mucedo* and *F. oxysporum* sp. *vasinfectum*. This is the first report on the biological 3α-O-acetyl conjugation of T-2 toxin and its derivatives. The acetyl conjugation of T-2 toxin was also observed in vegetative mycelia of some trichothecene non-producing fungal species (our unpublished results). The biological 3α-O-acetylation of trichothecenes, diaacetoxyscirpenol and 15-acetoxyscirpen-3,4-diol by trichothecene non-producing fungi of *Mucor mucedo* and *F. oxysporum* sp. *vasinfectum* was reported.

Thus, the author’s earlier findings on the 3-O-acetylation and the C-4 oxidation have been clearly explained by recent genetic researches. Although Tri8 is known to be involved in the C-3 deacetylation, the molecular genetic difference between 3-ADON- and 15-ADON-producers remains still uncertain.

### 8. Development of ELISA for Screening DON and NIV

Detection of trichothecene for food safety assurance has been markedly improved in the last decade. Technological advances in detection include the development of HPLC, GC-MS, LC-MS and ELISA. In the last few years, ELISA and ELISA-based procedures have been accepted because they offer the advantage of specificity, sensitivity, simplicity, and rapidity, which are important for routine testing. Although several ELISAs for DON detection have been developed utilizing monoclonal and polyclonal anti-DON antibodies, there are only a few reports in the literature concerned with the application of immunochemical methods to NIV detection. Considering the importance of co-occurrence
of DON and NIV in the viewpoint of food safety assurance, an ELISA-based system for detection of either NIV alone or in combination with DON in cereal samples was attempted to be developed.

8.1 Production of Monoclonal Antibodies against 3,4,15-TANIV and 3,15-DADON

Balb/C mice were immunized with 4,15-DANIV-3-O-hemisuccinate conjugated to keyhole limpet hemocyanin, and from the mice, 21 hybridomas secreting MAB which reacted with 3,4,15-TANIV were isolated. These MBAs were characterized in detail using 23 trichothecene derivatives, and classified into two distinct types of reactivity against analogues of NIV and DON. Two MBAs, KTM-205 and 208, were highly specific for 3,4,15-TANIV, whereas KTM-233, 239 and 240, cross-reacted with both 3,15-DADON and 3,4,15-TANIV to the same extent. However, these MBAs showed no reactivity against trichothecenes lacking the 8-keto function (e.g. T-2, DAS and their derivatives) and non-acetylated 8-ketotrichothecene (DON and NIV).

Because the hydroxyl group at the C-7 position of both NIV and DON is recalcitrant to acylation, the toxins are easily converted to the corresponding partial acetylation products (i.e. 3,4,15-TANIV and 3,15-DADON), which are readily detected by the MBAs developed in this study. The competitive indirect ELISAs for measuring NIV and DON were developed using these MBAs. The quantifiable level of 3, 4, 15-TANIV and 3, 15-DADON was about 0.3 to 1000 pg/mL in buffer by the indirect competitive ELISA with KTM-240. Thus, the two types of MBAs developed showed specific reactivity against 3, 4, 15-TANIV alone or in combination with 3,15-DADON. The MBAs will be useful for the rapid and routine screening of samples contaminated with NIV and/or DON by partial acetylation of these toxins.

8.2 Practical ELISA Kits for Measuring DON, NIV, and T-2/HT-2 toxin

Following the previous study on the development of ELISA for screening DON and NIV, the authors have developed and tested an ELISA system for individual measurement of DON, NIV, and T-2 plus HT-2 toxin using MBAs for 3, 4, 15-TANIV, for both 3, 4, 15-TANIV and 3, 15-DADON, and for acetyl-T-2 toxin. The assay system comprised three kits (designated the DON plus NIV kit, the NIV kit, and the T-2 plus HT-2 kit). The practical performance of the ELISA system was assessed by assaying trichothecene mycotoxins in wheat kernels. The ELISA system meets all the requirements for use in a routine assay in terms of sensitivity (detection limit: DON 80 ng/g, NIV 80 ng/g, T-2 toxin 30 ng/g), reproducibility (total coefficient of variation: 1.9 – 6.2%), accuracy (recovery: 93.8 – 112.0%), simplicity and rapidity (time required:<2 hr), mass handling (>42 samples/assay), and a good correlation with GC-MS (r =0.9146–0.9991). Components derived from the wheat extract did not interfere with the assay kits. The ELISA system is a useful alternative method to GC-MS, LC-MS, or LC-ultraviolet absorption for screening cereals and foods for trichothecene mycotoxin contamination.

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