APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1990, p. 3723–3726
0099-2240/90/123723-04$02.00/0

Vol. 56, No. 12

Comparative Study on the Natural Occurrence of *Fusarium* Mycotoxins (Trichothecenes and Zearalenone) in Corn and Wheat from High- and Low-Risk Areas for Human Esophageal Cancer in China

Y. LUO, T. YOSHIZAWA,* AND T. KATAYAMA

Department of Bioresource Science, Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-07, Japan

Received 4 June 1990/Accepted 1 October 1990

A comparative study on the natural occurrence of *Fusarium* toxins was done with 47 corn and 30 wheat samples collected in 1989 from Linxian and Shangqiu Counties in Henan Province, People's Republic of China, high- and low-risk areas, respectively, for esophageal cancer. Three trichothecenes (deoxynivalenol [DON], 15-acetyldeoxynivalenol [15-ADON], and nivalenol [NIV]) and zearalenone (ZEA) were detected in corn samples, and DON, NIV, and ZEA were found in wheat samples. Compared with Shangqiu corn, the incidence and mean level of DON in Linxian were 2.4 and 5.8 times higher, respectively, and those of 15-ADON were 16.3 and 2.6 times higher, respectively. The incidence and level of trichothecenes in wheat samples were significantly lower than those in corn. However, the level of DON in Linxian wheat was 3.3 times higher than in Shangqiu wheat. This is the first report indicating a significant difference in the natural occurrence of *Fusarium* toxins in main staple foods between high- and low-risk areas for esophageal cancer in the People's Republic of China.

Scab in wheat and barley and rot in corn are caused by species of *Fusarium* and result in not only heavy economic losses but also several toxic effects on humans and domestic animals following consumption of damaged cereals in the People's Republic of China. Several investigators have reported the natural occurrence of *Fusarium* mycotoxins, including deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA), as one of the causative agents of toxification of Chinese wheat and corn (10, 11, 16). In addition, some kinds of *Fusarium* mycotoxins have been suspected of involvement in human chronic mycotoxicoses, such as esophageal cancer and Kashin-Beck disease, in the People's Republic of China (6, 9, 14, 18).

The main high-risk areas for esophageal cancer and esophagitis in the People's Republic of China are located in three provinces, i.e., Henan, Hebei, and Shanxi. Linxian County in Henan Province is well-known as the highest-risk area. Its annual death rate due to esophageal cancer is 132 per 100,000, 8.4 times higher than that of Shangqiu County, a low-risk area in Henan Province which lies about 250 km southeast of Linxian. Studies on esophagitis and esophageal cancer in Linxian have suggested that esophageal carcinogenesis is most likely a multistage and multifactorial process in which deficiencies of certain vitamins and trace elements, high levels of nitrates and nitrites in food and drinking water, *Candida* esophagitis, and *Fusarium* mycotoxins might be involved (4–6, 8).

Contamination of staple foods by *Fusarium* species in five high cancer risk counties was significantly higher than that in three low-risk counties in Henan Province (21). Recently, appreciable amounts of five kinds of *Fusarium* mycotoxins were found in Linxian corn samples collected from esophageal cancer patients' families (7). Among these toxins, DON has been suggested as a clastogenic and potential in vivo tumor-promoting toxin (7, 17). In addition, some Chinese scientists have suggested that trichothecenes have a role in human esophageal carcinogenesis (6–8). However, no comparative study on the incidence and levels of *Fusarium* mycotoxins in high- and low-risk areas for esophageal cancer in the People's Republic of China has been reported. In this report, we described the natural occurrence of the known mycotoxins trichothecenes and ZEA in corn and wheat samples collected in Linxian in comparison with that in samples from Shangqiu.

MATERIALS AND METHODS

Chemicals. Trichothecene mycotoxins, including DON, NIV, 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), 3,15-diacetyldeoxynivalenol, ZEA, T-2 toxin (T-2), and HT-2 toxin, were individually dissolved in methanol at a concentration of 1 mg/ml and stored in a freezer. Trimethylsilylating reagent was prepared at an *N*-trimethylsilylimidazole–*N*,*O*-bis(trimethylsilyl)acetamide–trimethylchlorosilane ratio of 3:3:2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Corn and wheat grain samples. A total of 47 corn and 30 wheat samples (at least 250 g each) were collected in Linxian and Shangqiu Counties, Henan Province, in 1989. Among these, 27 corn and 15 wheat samples were collected from 27 different families of patients randomly selected in Linxian County. The remaining 20 corn and 15 wheat samples were from 20 different peasant families with no patients with esophageal cancer also randomly selected in Shanqiu.

Extraction and cleanup. Cereal samples were extracted by a slight modification of a previously published procedure (15). To 40 g of a finely ground sample, 160 ml of acetonitrile-water (3:1) was added and extracted for 30 min with a shaker. An 80-ml sample of the filtrate was defatted with *n*-hexane, followed by concentration to dryness. The residue was dissolved in 2 ml of methanol and transferred onto a Florisil column (2-cm [inside diameter] glass column; 10 g of Florisil was packed in *n*-hexane topped with 5 g of anhy-

* Corresponding author.

TABLE 1. Natural occurrence of trichothecenes and ZEA in corn from Linxian and Shangqiu[a]

Location (no. of samples)[b]	No. (%) positive for:		Mean (range) mycotoxin level (ng/g) in samples positive for:			
	Trichothecenes	ZEA	DON	15-ADON	NIV	ZEA
Linxian (27)	26 (96.3)	16 (59.3)	574[c] (17–3505)	274[c] (44–752)	11 (4–53)	44 (14–169)
Shangqiu (20)	8 (40.0)	1 (5.0)	99 (11–612)	104	ND[d]	39

[a] The trichothecenes DON, 15-DON, and NIV were quantified by GC-electron capture detection, and the results were confirmed by GC-mass spectrometry or thin-layer chromatography. ZEA was quantified by high-performance liquid chromatography with a spectrofluorometer.

[b] All Linxian samples were randomly collected in 1989 from the families of patients with esophageal cancer, whereas Shangqiu samples were also randomly collected from peasant families with no esophageal cancer.

[c] $P < 0.01$ by Student's t test.

[d] ND, Not detected.

drous sodium sulfate). The column was washed with 100 ml of *n*-hexane, followed by elution with 100 ml of chloroform-methanol (9:1). The eluate was evaporated to dryness under reduced pressure. The residue was redissolved in 2 ml of methanol to afford a sample solution.

Quantitation and confirmation of trichothecenes. A portion of a sample solution was reacted with the trimethylsilylating reagent, followed by water treatment by a previously published procedure (13), and analyzed with a gas chromatograph (GC) with a ^{63}Ni electron capture detector. The following conditions were used for the analysis: a Hitachi 263 gas chromatograph; a glass column (1 m by 3 mm [inside diameter]) packed with 2% OV-17 on 80/100-mesh Gas Chrom Q; column temperatures, 200°C for DON, NIV, and their derivatives and 240°C for T-2 toxin and its derivatives; detector and injector temperature, 270°C; nitrogen flow rate, 55 ml/min. Calculation of trichothecene concentrations was based on external standards of DON, 15-ADON and NIV, which eluted at retention times (t_R) of 4.6, 9.8, and 7.2 min, respectively.

To determine the presence of acetylated DON, a portion of a sample solution was mixed with an equal volume of 0.1 N aqueous NaOH and allowed to stand for 1 h at room temperature. Reaction products were derivatized to trimethylsilylating ethers and analyzed by GC-electron capture detection.

For confirmation of trichothecenes, a JEOL DX300 mass spectrometer equipped with a JMA-DA 5000 mass data system was used. Analytical conditions were as follows: column, Shimadzu Hicap CBP-1 fused silica capillary column (50 m by 0.2 mm [inside diameter]; Shimadzu Scientific Instruments, Inc., Kyoto, Japan) coated with methyl silicone (chemically bonded type); column temperature, 5 min at 160°C and then increased to 270°C at 4°C/min; injector temperature, 300°C; ion source temperature, 300°C; ionizing voltage, 70 eV; ionizing current, 300 μA; scanning rate, one scan per second.

High-performance liquid chromatography for ZEA. A Shimadzu LC-6A high-performance liquid chromatograph equipped with an FP-110 spectrofluorometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan) was used to identify ZEA. The column was a TSKgel ODS-80 (100 by 4.6 mm [inside diameter]; particle size, 5 μm; TOSOH, Tokyo, Japan). The emission and excitation wavelengths were set at 450 and 314 nm, respectively. The mobile phase was 70% aqueous methanol at a flow rate of 1 ml/min. Calculation of ZEA concentrations was based on an external standard of the toxin.

RESULTS

Natural occurrence of trichothecenes and ZEA in corn samples from Linxian and Shanqiu. By the analytical procedure described in Materials and Methods, recovery studies were performed on control corn samples spiked with 200 ng each of DON, NIV, T-2, and ZEA per g. Mean recoveries were 97 to 103% for the trichothecenes and 92% for ZEA. The limits of detection were estimated to be around 5 ng/g for DON, NIV, and their acetylated derivatives, 100 ng/g for T-2 and HT-2 toxins, and 5 ng/g for ZEA.

The natural occurrence of trichothecenes (DON, 15-ADON, and NIV) and ZEA in Linxian and Shangqiu corn samples is shown in Table 1. Neither 3-ADON nor T-2 was detected by GC-electron capture detection. The incidences of contaminated samples were 96.3% for DON, 81.5% for 15-ADON, 85.2% for NIV, and 59.3% for ZEA. The four toxins were coincidently found in 13 (48.1%) of 27 corn samples from Linxian, the three trichothecenes were in 19 (70.4%) of the corn samples, and DON and 15-ADON were in 21 (77.8%) of the corn samples. In addition, the level of 15-ADON was positively correlated with that of DON (r = 0.935), although the former was lower than the latter.

As for the incidence of corn contamination at a concentration of over 1,000 ng/g, four samples (14.8%) were contaminated with DON and nine (33.3%) were contaminated with total DON (DON plus 15-ADON). The maximal levels found in Linxian corn were 3,505 ng/g for DON, 752 ng/g for 15-ADON, and 169 ng/g for ZEA.

On the other hand, of 20 Shangqiu corn samples, 8 (40%) were contaminated with DON at a mean concentration of 99 ng/g. Although the maximal level of DON was 612 ng/g, the DON level in other Shangqiu corn was less than 50 ng/g. Both 15-ADON and ZEA were found in only one sample. None of the remaining mycotoxins was detected.

Natural occurrence of trichothecenes and ZEA in wheat grains from Linxian and Shangqiu. Recovery studies were performed with wheat samples just as with corn samples. Mean recoveries were 96.4% for DON, 103% for NIV, and 97% for ZEA at the same concentrations as those in spiked corn. The limits of detection for these mycotoxins were estimated to be the same as those in corn samples.

The natural occurrence of trichothecenes and ZEA in Linxian and Shangqiu wheat samples is summarized in Table 2. Among 30 wheat samples, either DON or ZEA was found at the same incidence in both Linxian and Shangqiu samples. 15-ADON was not detected. The mean concentrations of DON in positive samples were 59 ng/g in Linxian wheat and 18 ng/g in Shangqiu wheat, and the level of ZEA was less than 10 ng/g in both samples. NIV was detected at low concentrations, ranging from 13 to 21 ng/g in only 7 of 15 Shangqiu samples. It was not found at all in Linxian samples.

Confirmation of trichothecenes by GC and GC-mass spectrometry. Individual corn extracts from 14 Linxian samples contaminated with both DON and 15-ADON at a level above 100 ng/g were treated with 0.1 N aqueous NaOH. The GC

TABLE 2. Natural occurrence of trichothecenes and ZEA in wheat from Linxian and Shangqiu[a]

Location (no. of samples)[b]	No. (%) of samples positive for:		Mean (range) mycotoxin level (ng/g) in samples positive for:		
	Trichothecenes	ZEA	DON	NIV	ZEA
Linxian (15)	7 (46.7)	6 (40.0)	59[c] (9–309)	ND[d]	Tr[e]
Shangqiu (15)	7 (46.7)	6 (40.0)	18 (7–36)	15 (13–21)	Tr

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] $P < 0.05$ by Student's *t* test.
[d] ND, Not detected.
[e] Tr, Trace (below 10 ng/g).

peak corresponding to 15-ADON (t_R, 9.8 min) in all contaminated corn extracts completely disappeared after the treatment, whereas that of DON (t_R, 4.6 min) increased in height (Fig. 1). The peak corresponding to NIV (t_R, 7.2 min) was not affected by this treatment. The mean concentrations of DON and 15-ADON in 14 samples before hydrolysis were 978 (range, 121 to 3,505) and 413 (range, 142 to 752) ng/g, respectively, whereas the mean DON concentration was 1,371 (range, 247 to 4,098) ng/g after hydrolysis. The average percentage of transformation of 15-ADON into DON was calculated as 97% (range, 84 to 118%).

DON and 15-ADON in Linxian corn samples were confirmed by GC-mass spectrometry (Fig. 2). The mass spectrum of 15-ADON gave the diagnostic ions at *m/z* 467, 407, 392, and 350, although its molecular ion peak (*m/z* 482) overlapped with background peaks. The peaks at *m/z* 377, 363, and 362 found with standard 3-ADON were absent with 15-ADON, whereas those found at *m/z* 407 and 350 with 15-ADON were absent with 3-ADON. Thus, the occurrence of 15-ADON in Linxian samples was confirmed by comparing the mass fragmentions of the two toxins in addition to the retention times on GC. The occurrence of NIV in Linxian corn samples was confirmed by GC-mass spectrometry-selected ion monitoring.

DISCUSSION

The analytical results in Table 1 indicate that corn samples collected from Linxian were highly contaminated with the

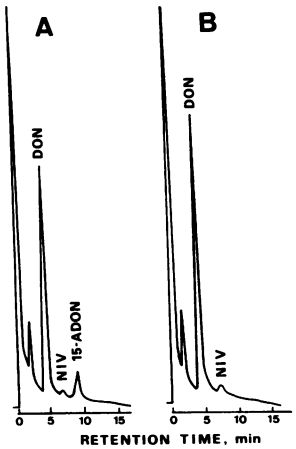

FIG. 1. Gas chromatograms of 15-ADON, DON, and NIV in Linxian corn extract (L-C-6H) before (A) and after (B) alkaline hydrolysis.

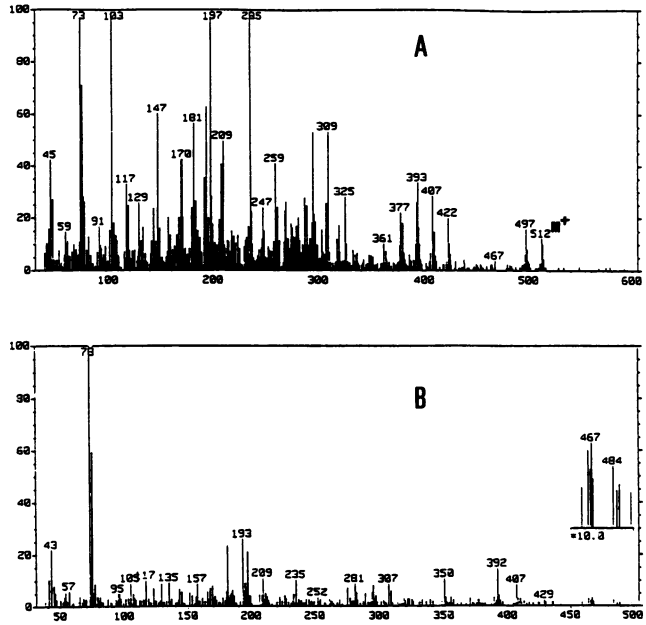

FIG. 2. Mass spectra of trimethylsilyl ether derivatives of DON (A) and 15-ADON (B) in Linxian corn.

Fusarium mycotoxins DON, 15-ADON, NIV, and ZEA. Among these toxins, the major mycotoxins in Linxian corn were DON and 15-ADON rather than NIV and ZEA. Compared with corn from Shangqiu, the incidence and mean levels of the major toxins in Linxian corn were much higher ($P < 0.01$), i.e., 2.4 and 5.8 times higher for DON and 16 and 2.6 times higher for 15-ADON, respectively. On the other hand, 15-ADON was not detected in wheat samples and the incidence and levels of the other trichothecenes in wheat samples from both areas were significantly lower than those in corn samples ($P < 0.05$). However, as with corn, the DON level of Linxian wheat was 3.3 times higher than that of Shangqiu wheat ($P < 0.05$) (Table 2). These results suggest that main staple foods, especially corn, consumed in the high-risk area are more frequently and highly contaminated with trichothecenes than are those consumed in the low-risk area.

Of 27 corn samples from Linxian, 33% were contaminated with total DON (DON plus 15-ADON) at levels above 1,000 ng/g (the tolerance level for DON in Canada) and as high as 48.1% were above 500 ng/g (the tolerance level in the Soviet Union). In comparison with the 50% lethal dose of DON for mice (2, 20), the estimated acute toxicity of 15-ADON is greater by the oral route (2). However, there is no tolerance for 15-ADON in any country. Much attention should be devoted to the natural occurrence of 15-ADON together with DON and the combined effects of these toxins on human health.

No definitive evidence indicating the involvement of DON and its derivatives in carcinogenesis is available. However, there are several reports suggesting this possibility for trichothecenes. Hsia et al. (6) speculated that trichothecenes could have a role in human esophageal carcinogenesis because the pattern of hyperplastic and dysplastic lesions induced by T-2 toxin in cultured fetal esophagus tissue was very similar to the morphology of premalignant lesions seen in the esophageal epithelia adjacent to human cancers. Mixtures of trichothecenes were shown to promote esopha-

geal tumors initiated in animals by a nitrosamine (17). Recently, Jone et al. (9) have reported that DON has the ability to inhibit metabolic cooperation, a measurement of gap junctional intracellular communication in the V_{79} cell system, suggesting that the toxin is a potential in vivo promoter. The differences in the incidence and levels of trichothecenes between high- and low-risk areas found in the present study provide additional support for the possible involvement of these mycotoxins in esophagitis and esophageal cancer in the People's Republic of China.

In contrast to the results reported by Hsia et al. (7), 3-ADON was not detected in any samples in the present study. By the analytical procedure described here, 3-ADON was detected together with DON, NIV, and ZEA in Japanese wheat and barley (T. Yoshizawa and Y. Luo, unpublished data). Abbas et al. (1) also reported the natural occurrence of 15-ADON, as well as DON and ZEA, in corn associated with feed refusal by swine in the United States. These results suggest a geographic difference in the occurrence of monoacetyl isomers of DON in cereals (12). In addition, the high correlation between levels of DON and 15-ADON in Linxian corn ($r = 0.935$) indicates that both toxins have the same fungal origin and are probably produced by a subclassified type of 15-ADON-producing *Fusarium gaminearum* (19). Furthermore, the possibility of simultaneous infection of Linxian corn samples with other *Fusarium* species and resulting co-occurrence of trichothecenes with other toxins produced by these species should not be ruled out.

This is the first report of a comparative study of the natural occurrence of *Fusarium* mycotoxins in main staple foods with specific reference to human esophageal cancer in the People's Republic of China. Further comparative studies are needed to clarify the yearly changes of the incidence and levels of trichothecenes in main staple foods in high-risk areas for esophageal cancer and also to survey other clastogenic *Fusarium* mycotoxins, such as fumonisins (3).

ACKNOWLEDGMENTS

This work was supported in part by the Japanese and Chinese Medical Association.

We thank J. S. Yang and H. Li, Institute of Pharmacology and Toxicology, Beijing, People's Republic of China, for providing cereal samples from Linxian and Shangqiu Counties.

LITERATURE CITED

1. **Abbas, H. K., C. J. Mirocha, and J. Tuite.** 1986. Natural occurrence of deoxynivalenol, 15-acetyldeoxynivalenol, and zearalenone in refusal factor corn stored since 1972. Appl. Environ. Microbiol. **51**:841–843.
2. **Forsell, J. H., R. Jensen, J. H. Tai, M. Wilt, W. S. Lin, and J. J. Pestka.** 1987. Comparison of acute toxicities of deoxynivalenol (vomitoxin) and 15-acetyldeoxynivalenol in the $B_6C_3F_1$ mouse. Food Chem. Toxicol. **25**:155–162.
3. **Gelderblom, W. C. A., K. Jaskewicz, W. F. O. Marasas, P. G. Thiel, R. M. Horak, R. Vleggaar, and N. P. J. Kriek.** 1988. Fumonisins—novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Appl. Environ. Microbiol. **54**:1806–1811.
4. **Hsia, C. C.** 1983. Possible roles of fungal infection and mycotoxins in human esophageal carcinogenesis, p. 883–911. *In* C. C. Harris and H. Autrup (ed.), Human carcinogenesis. Academic Press, Inc., New York.
5. **Hsia, C. C., Y. Gao, J. L. Wu, and B. L. Tzian.** 1986. Induction of chromosome aberrations by *Fusarium* T-2 toxin in cultured human peripheral blood lymphocytes and Chinese hamster fibroblasts. J. Cell. Physiol. Suppl. **4**:65–72.
6. **Hsia, C. C., B. L. Tzian, and C. C. Harris.** 1983. Proliferative and cytotoxic effects of *Fusarium* T-2 toxin on cultured human fetal esophagus. Carcinogenesis **4**:1101–1107.
7. **Hsia, C. C., J. L. Wu, X. Q. Lu, and Y. S. Li.** 1988. Natural occurrence and clastogenic effects of nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and zearalenone in corn from a high risk area of esophageal cancer. Cancer Detect. Prev. **13**:79–86.
8. **Hsia, C. C.** 1984. Carcinogenesis in the esophagus, p. 53–76. *In* G. J. Huang and Y. K. Wu. (ed.), Carcinoma of the esophagus and gastric cardia. Springer Verlag KG, Berlin.
9. **Jone, C., L. Erickson, J. E. Trosko, and C. C. Chang.** 1987. Effect of biological toxins on gap-junctional intercellular communication in Chinese hamster V_{79} cells. Cell Biol. Toxicol. **3**:1–15.
10. **Lu, G., Y. Xue, H. Z. Zhiu, M. F. Chen, and L. Li.** 1988. Investigation of deoxynivalenol and zearalenone in wheat from Anhui province. Proc. Jpn. Assoc. Mycotoxicol. Suppl. **1**:67–68.
11. **Luo, X. Y.** 1988. *Fusarium* toxins contaminations of cereals in China. Proc. Jpn. Assoc. Mycotoxicol. Suppl. **1**:97–98.
12. **Mirocha, C. J., H. K. Abbas, C. E. Windels, and W. Xie.** 1989. Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by *Fusarium graminearum* isolates. Appl. Environ. Microbiol. **55**:1315–1316.
13. **Rizzo, A. F., L. Saari, and E. Lindfors.** 1986. Determination of trichothecenes and water treatment of their trimethylsilyl ethers in an anhydrous apolar solvent. J. Chromatogr. **368**:381–386.
14. **Sokoloff, L.** 1985. Endemic forms of osteoarthritis. Clin. Rheum. Dis. **11**:187–202.
15. **Tanaka, T., A. Hasegawa, Y. Matsuki, K. Ishii, and Y. Ueno.** 1985. Improved methodology for the simultaneous detection of the trichothecene mycotoxins deoxynivalenol and nivalenol in cereals. Food Addit. Contam. **2**:125–137.
16. **Ueno, Y., T. Tanaka, A. Hasegawa, Z. H. Hu, and D. D. Xu.** 1986. Deoxynivalenol, nivalenol and zearalenone in Scabby wheat from Shanghai, China. J. Food Hyg. Soc. Jpn. **27**:180–182.
17. **Van Rensburg, S. J.** 1986. Role of mycotoxins in endemic liver and esophageal cancer, p. 483–494. *In* P. S. Steyn and R. Vleggaar (ed.), Bioactive molecules 1. Mycotoxins and phycotoxins. Elsevier, Amsterdam.
18. **Yang, J. B.** 1989. Study of cause of Kaschin-Beck disease and intervention measures. J. Chin. Local Dis. **8**:134–184. (In Chinese).
19. **Yoshizawa, T., and P. Luangpitsuksa.** 1985. Microbial transformation of trichothecene mycotoxins by deoxynivalenol-producing strains of *Fusarium graminearum*. Proc. Jpn. Assoc. Mycotoxicol. **21**:6–8.
20. **Yoshizawa, T., and N. Morooka.** 1974. Studies on the toxic substances in the infected cereals. Acute toxicities of new trichothecene mycotoxins: deoxynivalenol and its monoacetate. J. Food Hyg. Soc. Jpn. **15**:261–269.
21. **Zhen, Y. Z., W. X. Yang, S. L. Yang, Q. S. Liu, L. P. Ding, and F. M. Han.** 1984. The culture and isolation of fungi from the cereals in five high and three low incidence counties of esophageal cancer in Henan Province. J. Chin. Tumor **6**:27–29. (In Chinese).