

Variation in Trichothecene and Zearalenone Production by *Fusarium graminearum* Isolates from Corn and Barley in Korea

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A total of 110 *Fusarium graminearum* isolates were obtained from corn and barley samples which were collected from Kangwon province and the southern part of Korea, respectively. The isolates were tested for trichothecene and zearalenone (ZEA) production in rice culture. The incidences of trichothecene production by 51 isolates of *F. graminearum* from corn were 64.7% for deoxynivalenol (DON), 7.8% for 3-acetyldeoxynivalenol (3-ADON), 33.3% for 15-acetyldeoxynivalenol (15-ADON), 21.6% for nivalenol (NIV), and 13.7% for 4-acetylnivalenol (4-ANIV). DON producers frequently co-produced 15-ADON rather than 3-ADON. On the other hand, the incidences of trichothecene production by 59 isolates of *F. graminearum* from barley were 71.2% for NIV, 61.0% for 4-ANIV, and only one isolate produced DON and 3-ADON. The incidences and mean levels of ZEA producers were 32.0% and 71 µg/g for the isolates from corn, and 29.0% and 74 µg/g for the isolates from barley. There was a great regional difference in trichothecene production of *F. graminearum* isolates between Kangwon province and the southern part of Korea.

KEY WORDS □ *Fusarium graminearum*, trichothecene, zearalenone, toxigenic potential

Fusarium graminearum Schwabe, the imperfect stage of *Gibberella zeae* (Schw.) Petch, is a fungal pathogen of many cereals including corn, wheat, and barley. This fungus produces toxic metabolites such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), 4-acetylnivalenol (4-ANIV), and zearalenone (ZEA) (Fig. 1). These mycotoxins have been found in grains and animal feeds (2, 3, 9, 16, 19, 22, 24, 25) and are responsible for mycotoxicoses in farm and experimental animals (5, 6, 17, 20).

Based on the production of the different trichothecenes, Ichinoe *et al.* (8) reported that *G. zeae* is chemotaxonomically divided into two types; one is the NIV chemotype which produces NIV and 4-ANIV and the other is the DON chemotype which produces DON and 3-ADON. ZEA is produced by these two chemotypes. Lee *et al.* (11) and Logrieco *et al.* (13) have also reported the two chemotypes in *Fusarium* isolates from cereals in Korea and Italy, respectively. It appears that there are regional differences in the natural distribution of the toxigenic isolates as well as their corresponding trichothecenes. For example, the presence of the NIV chemotype was

reported in Korea, Japan, Taiwan, and Italy (1, 11, 13). However, the NIV chemotype was not reported in North American countries, such as Canada and the United States, although grain contamination with NIV was reported in these countries (23). North American isolates of *F. graminearum* are DON producers and DON producers co-produce 15-ADON rather than 3-ADON (15).

In previous papers (12, 21), we examined the incidence of *F. graminearum* in corn and barley from Kangwon province and the southern part of Korea, respectively. In this paper, we have carried out an extensive investigation of *F. graminearum* isolates from corn and barley to determine whether there was a correlation between the geographic origins of the isolates and toxin production.

MATERIALS AND METHODS

Chemicals

Trichothecene mycotoxins including DON, 3-ADON, 15-ADON, NIV, and 4-ANIV were obtained from Dr. T. Yoshizawa, Department of Bioresource Science, Faculty of Agriculture,

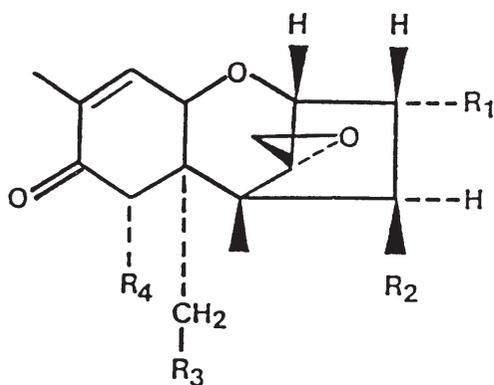


Fig. 1. Structure of trichothecenes produced by *Fusarium* species.

Deoxynivalenol has OH for R₁, H for R₂, OH for R₃, and OH for R₄.

3-Acetyldeoxynivalenol has OAc for R₁, H for R₂, OH for R₃, and OH for R₄.

15-Acetyldeoxynivalenol has OH for R₁, H for R₂, OAc for R₃, and OH for R₄.

Nivalenol has OH for R₁, OH for R₂, OH for R₃, and OH for R₄.

4-Acetylnivalenol has OH for R₁, OAc for R₂, OH for R₃, and OH for R₄.

Kagawa University, Japan. ZEA was purchased from Sigma Chemical Co. Each toxin was individually dissolved in methanol at the concentration of 1 mg/ml and stored in a freezer. Trimethylsilylating reagent was prepared with an N-trimethylsilylimidazole-*N,O*-bis(trimethylsilyl)acetamide-trimethylchlorosilane at ratio of 3:3:2 (Wako Pure Chemical Industries, Ltd.).

Corn and barley samples

A total of 39 cereal samples were collected. Fifteen corn samples were collected from 15 counties in Kangwon province during November in 1991. Twenty-four barley samples were collected from different farmers' stocks in Chonbuk, Chonnam, Kyungbuk, and Kyungnam provinces during July in 1992.

Isolation of *Fusarium* species

From each grain sample, 100 kernels were randomly selected, shaken in 2% NaOCl for 1 min, rinsed in sterile distilled water, and transferred to potato-dextrose agar plates followed by incubation at 25°C for 4~7 days. *Fusarium* species were transferred from grains to noncommercial potato-dextrose agar or carnation leaf agar (4), or both, incubated under fluorescent lamps (5,000 lux) at 25°C, and identified by using the manual of Nelson *et al.* (18). Because the *Fusarium* colonies isolated from the two cereal samples numbered into the hundreds, only *F. graminearum* isolates (110 isolates) were selected and assayed

for mycotoxins. Stock cultures of *F. graminearum* isolates were isolated in single spore. They were maintained on autoclaved soils and stored at -15°C.

Preparation of cultures

Erlenmeyer flasks (500 ml), each containing 100 g of rice and 60 ml of distilled water, were autoclaved twice for 1 h at 121°C with a 24-hour interval. The rice was inoculated with mycelium plugs from 5-day-old potato dextrose agar of each isolate. The flasks were incubated for 2 weeks at 25°C followed by 2 weeks at 10°C. The mycelial mass and substrate were disbursed onto a screen-bottom tray and allowed to air dry in a ventilated hood. When dry, this inoculated substrate was ground to the consistency of flour and stored at 15°C until analysis.

Extraction and clean-up

The rice cultures were extracted by the procedure reported previously (14). Each ground culture (40 g) was extracted with 160 ml of acetonitrile-water (3:1, v/v) for 30 min and the extract was filtered through Whatman No.1 filter paper. An 80-ml filtrate was defatted with the same volume of *n*-hexane and concentrated to dryness. The residue was dissolved in 2 ml of methanol and applied onto a Florisil column (2×15 cm) containing 10 g of Florisil (60~100 mesh, Fisher Scientific Co.). The column was washed with 100 ml of *n*-hexane, followed by elution with 100 ml of chloroform-methanol (9:1, v/v). The elute was concentrated to dryness and the residue was redissolved in 2 ml of methanol.

Quantitation and confirmation of trichothecenes

A portion of each extract was reacted with trimethylsilylating reagent and analyzed with a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector. The following conditions were used for the analysis: column, Ultra 2 capillary column (0.32 mm id by 25 m, 0.2 μm film thickness, Hewlett-Packard); carrier, nitrogen gas at a flow rate of 3 ml/min; column temperature, 5 min at 170°C and then increased 250°C at 6°C/min; injector temperature, 270°C; detector temperature, 290°C. Calculation of trichothecene concentration was based on external standards of DON, 3-ADON, 15-ADON, NIV, and 4-ANIV.

For the confirmation of trichothecenes, each culture extract was derivatized with the trimethylsilylating reagent and analyzed by a JEOL AX 505 mass spectrometer equipped with a MSMP-DAP-2 data system. The analytical conditions were as follows: column, DB-5 fused silica column (0.25 mm id by 30 m, 0.25 μm film thickness, J & W Scientific); column temperature, 160°C for 5 min and then increased to 270°C at 5°C/min; injector temperature, 270°C; ion source temperature, 200°C; ionizing voltage, 70 eV; ionizing current, 300 μA; scanning rate, 2 sec/scan.

Analysis of ZEA by high performance liquid chromatography (HPLC)

A Shimadzu LC-6A HPLC equipped with PF-110 spectrofluorometer (Japan Spectroscopic Co. Ltd.) was used for the analysis of ZEA. The analytical conditions were as follows: column, Zorbax ODS column (4.6 mm×15 cm, 5 µm particle size, Dupont Co.); mobile phase, 70% aqueous methanol; flow rate, 1 ml/min; excitation wavelength, 236 nm; emission wavelength, 418 nm. Calculation of ZEA concentration was based on an external standard of ZEA.

RESULTS

Trichothecene and ZEA production by *F. graminearum* isolates from corn

The production of 8-ketotrichothecenes (DON, 3-ADON, 15-ADON, NIV, and 4-ANIV) and ZEA by corn isolates of *F. graminearum* is summarized in Table 1. The incidences of trichothecene production by corn isolates were 64.7% for DON, 7.8% for 3-ADON, 33.3% for 15-ADON, 21.6% for NIV, and 13.7% for 4-ANIV. Of 51 isolates, 33 isolates were DON-type producers and 12 were NIV-type producers; DON and 15-ADON were produced by 17 isolates, DON and 3-ADON by 4 isolates, DON by 12 isolates, NIV and 4-ANIV by 5 isolates, NIV by 6 isolates, and 4-ANIV by 1 isolate. ZEA was frequently found with these trichothecene-producing isolates and 32 of 51 isolates were ZEA producers. The mean concentrations of the toxins detected in corn isolates were 170 (trace to 2,699) µg/g for DON, 31 (1.5 to 55) µg/g for 3-ADON, 10 (3.2 to 59) µg/g for 15-ADON, 5.2 (0.2 to 13) µg/g for NIV, 5.4 (1.0 to 11) µg/g for 4-ANIV, and 71 (trace to 431) µg/g for ZEA.

Trichothecene and ZEA production by *F. graminearum* isolates from barley

The production of trichothecenes and ZEA by barley isolates of *F. graminearum* is shown in Table 2. Major trichothecenes found in barley

Table 1. Production of mycotoxins by 51 isolates of *Fusarium graminearum* from corn.

Mycotoxins	No. (%) of positives	Mean (range) level in positives
DON	33 (64.7)	170 (trace-2,699) µg/g
3-ADON	4 (7.8)	31 (1.5-55) µg/g
15-ADON	17 (33.3)	10 (3.2-59) µg/g
NIV	11 (21.6)	5.2 (0.2-13) µg/g
4-ANIV	7 (13.7)	5.4 (1.0-11) µg/g
ZEA	32 (62.7)	71 (trace to 431) µg/g

The trichothecenes were quantified by capillary-GC with a flame ionization detector and ZEA was quantified by HPLC with a fluorescence detector.

Table 2. Production of mycotoxins by 59 isolates of *Fusarium graminearum* from barley.

Mycotoxins	No. (%) of positives	Mean (range) level in positives
DON	1 (1.6)	841 µg/g
3-ADON	1 (1.6)	830 µg/g
15-ADON	0 (0.0)	0 µg/g
NIV	42 (71.2)	43 (0.9-416) µg/g
4-ANIV	36 (61.0)	25 (0.9-352) µg/g
ZEA	29 (49.2)	74 (trace-1117) µg/g

The trichothecenes were quantified by capillary-GC with a flame ionization detector and ZEA was quantified by HPLC with a fluorescence detector.

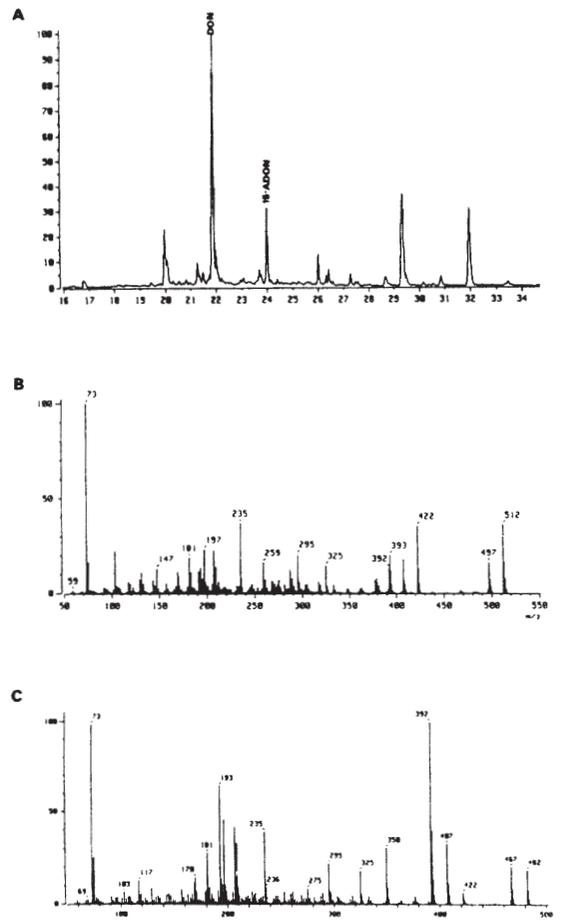


Fig. 2. Total ion chromatogram of an extract of *F. graminearum* culture (A) and mass spectra of TMS derivatives of DON (B) and 15-ADON (C) with retention times of 21.87 and 24.01 min, respectively.

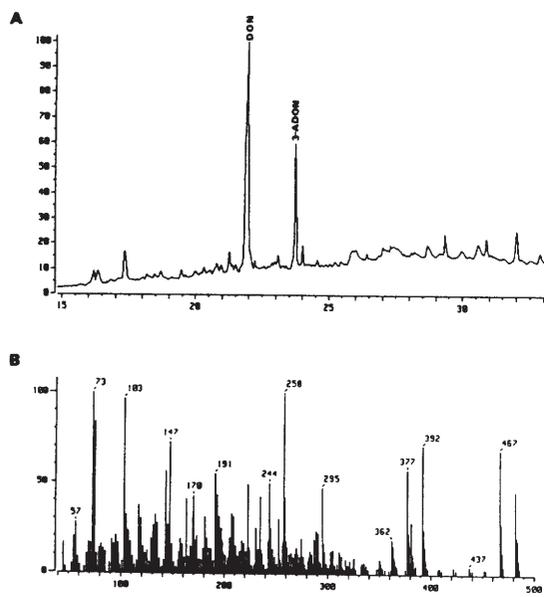


Fig. 3. Total ion chromatogram of an extract of *F. graminearum* culture (A) and a mass spectrum of TMS derivatives of 3-ADON (B) with a retention time of 23.77 min.

isolates were NIV-type; the incidences of trichothecene production were 71.2% for NIV and 61.0% for 4-ANIV. Of 59 isolates, 35 isolates co-produced NIV and 4-ANIV, 7 isolates produced NIV, and one isolate produced 4-ANIV. Only one isolate co-produced DON and 3-ADON at the level of 841 $\mu\text{g/g}$ and 826 $\mu\text{g/g}$, respectively. None of the barley isolates produced 15-ADON. In addition, ZEA was also found in 29 of 59 isolates. The mean concentrations of NIV, 4-ANIV, and ZEA found in barley isolates were 43 (0.9 to 416) $\mu\text{g/g}$, 25 (0.9 to 352) $\mu\text{g/g}$, and 74 (trace to 1117) $\mu\text{g/g}$, respectively.

Confirmation of trichothecenes by GC-MS

In order to verify the presence of trichothecenes unequivocally, three culture extracts which were positive for trichothecenes (DON plus 15-ADON, DON plus 3-ADON, and NIV plus 4-ANIV) were chosen and then subjected to capillary GC-MS.

Fig. 2A shows the total ion chromatogram of trimethylsilyl (TMS) derivatives of DON and 15-ADON with retention times of 21.87 and 24.01 min, respectively. The mass spectrum of DON-TMS gave diagnostic peaks at m/z 512, 497, 422, 325, 295, and 235 and a molecular ion peak at m/z 512 (Fig. 2B). The mass spectrum of 15-ADON-TMS gave diagnostic peaks at m/z 482, 350, 325, 295, 235, and 193 (Fig. 2C).

Fig. 3A shows the total ion chromatogram of TMS derivatives of DON and 3-ADON with retention times of 21.87 and 23.77 min. The major

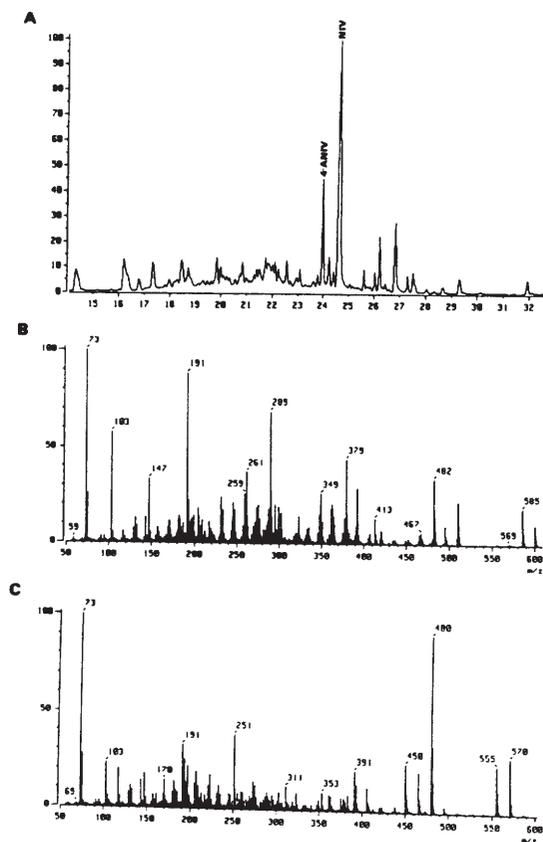


Fig. 4. Total ion chromatogram of TMS derivatives of an extract of *F. graminearum* culture (A) and mass spectra of TMS derivatives of NIV (B) and 4-ANIV (C) with retention times of 24.54 and 23.93 min, respectively.

fragmented pattern of 3-ADON-TMS (Fig. 3B) was similar to that of 15-ADON-TMS. The fragmented ions at m/z 377, 363, and 362 found in 3-ADON were absent in 15-ADON.

Fig. 4A shows the total ion chromatogram of NIV and 4-ANIV and mass spectra of the two trichothecenes. The major fragmented ions of NIV-TMS were m/z 600, 585, 482, 379, 349, and 289, and those of 4-ANIV-TMS were m/z 570, 555, 480, 465, 450, 391, and 251 (Fig. 4B and 4C).

DISCUSSION

In the present study, we examined the production of trichothecenes and ZEA of *F. graminearum* isolates from corn and barley. The data indicate the presence of two toxigenic groups of *F. graminearum* (DON chemotype and NIV chemotype) in Korea. Lee *et al.* (11) isolated *Fusarium* species from Korean cereals and also

found the two chemotypes in trichothecene production. DON producers did produce 3-ADON. None of the tested isolates produced 15-ADON.

There is a remarkable difference in trichothecene production of *F. graminearum* isolates from corn compared to those from barley in Korea. Among the corn isolates of *F. graminearum*, the incidence of DON producers was much higher than that of NIV producers. DON producers frequently co-produced 15-ADON, but DON and 3-ADON co-producers were rare. On the other hand, most of the barley isolates of *F. graminearum* were NIV producers and only one was a DON and 3-ADON co-producer. None of the isolates tested were found to produce 15-ADON. These chemotypes of trichothecenes in *Fusarium* isolates from corn and barley support the natural occurrence of trichothecenes in the two cereals (10). The major contaminants in corn samples were DON and 15-ADON and those in barley samples were NIV and DON.

At this moment, we may conclude that there is a regional difference in trichothecene production by *F. graminearum* isolates between Kangwon province and the southern part of Korea, which are the major corn- and barley-producing areas, respectively. Such regional difference in trichothecene production by *F. graminearum* isolates were also observed in Japan (7). However, host-selected difference in trichothecene production by *Fusarium* species should not be ruled out. In order to verify this hypothesis, it is necessary to examine corn and barley isolates of *F. graminearum* from the southern part of Korea and Kangwon province, respectively.

It should be also stressed that barley isolates produce greater quantities of NIV and 4-NIV than corn isolates. The wide distribution of NIV chemotypes being capable of producing great quantities of trichothecenes in the southern part of Korea might account for a portion of the disease syndrome occurred in 1963 (3).

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초 록: 한국산 옥수수 및 보리로 부터 분리한 *Fusarium graminearum* 균주의 Trichothecene과 Zearalenone 생성변이

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강원도와 남부지방에서 각각 옥수수와 보리를 채집하여 두 곡류로 부터 총 110 균주의 *Fusarium graminearum*을 분리한 후 쌀배지에서의 trichothecene과 zearalenone(ZEA)의 생성능을 측정하였다. 옥수수로 부터 분리한 51개의 *F. graminearum* 균주중 trichothecene 생성빈도는 deoxynivalenol (DON)이 64.5%, 3-acetyldeoxynivalenol (3-ADON)이 7.8%, 15-acetyldeoxynivalenol (15-ADON)이 33.3%, nivalenol(NIV)이 21.6%, 4-acetylnivalenol(4-ANIV)이 13.7%였다. DON을 생성하는 균주들은 3-ADON보다는 15-ADON을 동시에 생성하였다. 한편, 보리로 부터 분리한 59개의 *F. graminearum* 균주중 trichothecene 생성빈도는 NIV가 71.2%, 그리고 4-ANIV가 61.0%였으며, 한 균주만이 DON과 3-ADON을 동시에 생성하였다. 옥수수로 부터 분리한 균주들의 ZEA의 생성빈도와 생성량은 각각 32.0%, 71 µg/g였으며, 보리로 부터 분리한 균주는 각각 29%, 745 µg/g이었다. 이와같이 우리나라 강원도지방과 남부지방의 *F. graminearum* 균주들의 trichothecene 생성양상은 지역적인 큰 차이를 나타내었다.