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Production of T-2 Toxin and Its Metabolites by *Fusarium sporotrichioides* Isolates from the Corn Producing Area in Korea

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우리나라 옥수수산지에서 분리한 *Fusarium sporotrichioides* 균주들에 의한 T-2 독소 및 관련 대사물의 생성

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ABSTRACT: Four isolates of *Fusarium sporotrichioides* obtained from the corn producing area were tested for their toxicities by feeding the crude cultures to rats. Three out of four isolates were highly toxic and killed all rats within 3-4 days after feeding. The chemical analyses of toxic cultures by thin layer chromatography and gas chromatography-mass spectrometry revealed that two isolates from Jeongsun district produced T-2 toxin and its related trichothecenes. This is the first report that *F. sporotrichioides* isolates produce T-2 toxin in Korea.

KEYWORDS: Trichothecene production, *Fusarium sporotrichioides*, T-2 toxin

T-2 toxin(3-hydroxy-4,15-diacetoxy-8-[3-methylbutyryloyl]-12,13-epoxytrichothec-9-ene) and its related trichothecenes are known to be produced by isolates of different *Fusarium* species (Marasas *et al.*, 1984) and possibly by *Trichoderma* species (Bamburg and Strong, 1969). However the major producers of T-2 toxin are confined to *F. sporotrichioides* (Marasas *et al.*, 1987). *F. sporotrichioides* is a fungus widespreaded on many plants and in soil throughout the cold and cold-temperate regions of the world.

At least two mycotoxicoses, alimentary toxic aleukia in Soviet Union (Joffe, 1971) and beanhulls poisoning of horses in Japan (Ueno *et al.*, 1972) are known to be associated with the ingestion of moldy grains and beans infected with T-2 producing isolates of *F. sporotrichioides*. Dermal necrosis, hemorrhaging, refusal of feeds, and bone marrow depression occurred in man and farm animals after consuming moldy grains and beans.

In the preceding paper (Lee *et al.*, 1988), we reported that the corns produced in Korea were highly contaminated with *Fusarium* species and many of the *Fusarium* isolates from corn and soil were toxic to rats. Lee *et al.* (1985; 1986) reported that Korean cereals were contaminated with trichothecenes (deoxynivalenol and nivalenol) and zearalenone (ZEN). Most *Fusarium* isolates obtained from cereals produced in Korea produced nivalenol, deoxynivalenol, fusarenone-X, 3-acetyl-deoxynivalenol or ZEN (Lee *et al.*, 1986). However, T-2 toxin was not produced by those *Fusarium* isolates.

During the chemical analyses of toxic cultures of *Fusarium* isolates obtained from the corn-producing area in Korea, we found that *F. sporotrichioides* could produce T-2 toxin and its

related trichothecenes. Therefore we report here our examination of several *F. sporotrichioides* isolates and the identification of trichothecenes in cultures grown on wheat.

Materials and Methods

Standard mycotoxins and chemicals

Trichothecene standards including T-2 toxin, diacetoxyscirpenol, fusarenone-X, monoacetoxyscirpenol, deoxynivalenol, and nivalenol were kindly provided by Dr. C.J. Mirocha, Department of Plant Pathology, University of Minnesota, U.S.A. 3-Acetoxy-deoxynivalenol was a gift from Dr. T. Yoshizawa, Department of Bioresources, Faculty of Agriculture, Kagawa University, Japan.

4-(*p*-nitrobenzyl) pyridine (NBP) and tetraethylenepentamine (TEPA) were purchased from Sigma Chemical Co., St. Louis, Mo, U.S.A.. *p*-Anisaldehyde was obtained from Aldrich Chemical Co., Milwaukee, Wi, U.S.A.. All organic solvents used were reagent grade unless otherwise noted.

Sources of *Fusarium* cultures

The sources of *F. sporotrichioides* cultures used in this study were described earlier by Lee *et al.* (1988), and were obtained principally from Kangwon province, where is the major corn producing area in Korea. Species were identified as described by Nelson *et al.* (1983).

Fungal cultures

Four isolates of *F. sporotrichioides* were cultured on an autoclaved wheat medium, described as a modification of the procedure developed by Lee and Mirocha (1984). In brief, 200g of wheat and 120 m*l* of distilled water were allowed to stand for 1 hr in a 1-liter flask, then autoclaved for 1 hr at 121°C twice with a 24 hrs interval. Flasks containing this medium were inoculated with *F. sporotrichioides* isolates and incubated at room temperature (22 to 26°C) for two weeks, with daily shaking for the first week to provide uniform growth of mycelium throughout the substrate. These flasks were then transferred to an incubator at 10 to 12°C for two weeks. After four weeks, the mycelial mass and substrate were disbursed onto a screen-bottom tray and allowed to air dry in a ventilated hood. When dry, the cultures were ground to the consistency of flour and used to feed rats and analyze the mycotoxins.

Toxicity test

Twenty-one-day-old virgin female Sprague-Dawley rats (50 to 60g) were obtained from the Experimental Animal Farm, Seoul National University. The rats were housed in individual cages and fed a 1:1 mixture (wt/wt) of ground moldy wheat and a complete rat diet. The complete rat diet consisted of following ingredients:mineral 175g, sucrose 525g, vitamins 50g, corn oil 200 m*l*, casein 600g; and ground wheat grain 950g. This was mixed until corn oil and other ingredients were well distributed. Then 2,500g of ground wheat grain were added and mixed thoroughly to give 5,000g. Rats were weighed at the start and end of the experiment, and three rats per *Fusarium* isolate were tested. Three control rats were fed a 1:1 mixture of ground, autoclaved wheat and a complete rat diet. After 5 days, the rats were killed and examined for gross pathological changes in organs and tissues.

Extraction of cultures

Fifty grams of ground wheat culture were added 15 m*l* of distilled water and extracted three times with 150 m*l* of methanol. The extract was filtered through Whatman No. 1 filter paper. The filtrates were combined and concentrated *in vacuo*. This crude extract was dissolved in 5 m*l* of methanol, and 50 m*l* of distilled water was added. The methanol-water azeotrope was concentrated until only water phase remained. The water phase was applied to a column (1.5 by 15 cm) packed with washed Amberlite XAD-2 resins (10g, 20-50 mesh, Sigma Chemical Co., St. Louis, U.S.A.). The column was rinsed with 100 m*l* of distilled water and eluted with 100 m*l* of 90% aqueous methanol. The methanol eluate was evaporated to dryness *in vacuo*, dissolved in 5 m*l* of chloroform-methanol (9:1, vol/vol), and applied to a Florisil column (10g, 1.5 by 15 cm, 60-100 mesh; Fisher Scientific Co., Pittsburgh, Pa, U.S.A.), which was topped with 3g of anhydrous sodium sulfate. The column was washed with 100 m*l* of *n*-hexane and eluted with 100 m*l* of chloroform-methanol (9:1, vol/vol). The eluate was dried and redissolved in 2 m*l* of

methanol.

Thin layer chromatography (TLC)

The culture extracts were analyzed for trichothecenes by TLC. Usually 10 μl of each extract was chromatographed onto silica gel 60 plates (0.25 mm thickness, E. Merck, AG. Darmstadt, Federal Republic of Germany) with the developing solvent systems of chloroform-methanol (9:1, vol/vol), chloroform-acetone (3:2, vol/vol), and ethyl acetate-toluene (3:1, vol/vol). The reagents used to detect trichothecenes on thin-layer chromatographic plates were 20% sulfuric acid in methanol, *p*-anisaldehyde solution (Pathre and Mirocha, 1977), and NBP-TEPA described by Takitani *et al.* (1974), which give trichothecenes a blue color.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was carried out on a Hewlett-Packard 5987B GC-MS equipped with an updated computerized data system. The culture extract was reacted at room temperature with trifluoroacetic anhydride (Pierce Chemical Co., Rackford, Ill. U.S.A.) to form trifluoroacetate (TFA) derivatives. After the sample and trifluoroacetic anhydride were mixed, the solution was heated for 30 min. at 45°C. The solution was then dried under nitrogen, and 50 μl of toluene was added before the solution was injected into GC-MS. Injection (1 μl) was into a CPDB-5 covalent bonded capillary column (3 m by 0.25 mm). A temperature program from 150°C to 280°C (10°C/min) was used with injector temperature at 300°C and the detector set at 200°C, and the helium (10 psi) was used as carrier gas.

Mass spectrum of each peak derived from GC was obtained as electron impact and identification of trichothecenes was done by comparison of observed spectra of known trichothecenes in data base library.

Table I. Thin layer chromatographic separation of various trichothecene standards.

Trichothecene	Rf value		
	A	B	C
T-2 toxin	0.70	0.53	0.44
Diacetoxyscirpenol	0.66	0.51	0.42
3-Acetoxydeoxynivalenol	0.58	0.45	0.34
Fusarenone-X	0.46	0.30	0.25
Monoacetoxyscirpenol	0.39	0.23	0.17
Deoxynivalenol	0.31	0.20	0.16
Nivalenol	0.11	0.04	0.02

Solvent systems were: A) chloroform-methanol (9:1, vol/vol), B) chloroform-acetone (3:2, vol/vol), and C) ethyl acetate-toluene (3:1, vol/vol).

Results and Discussion

Toxicity test

A total of four cultures of *F. sporotrichioides* were tested for toxicity by feeding a 1:1 mixture of fungal culture and a basal diet with the following observation: body weight change; feed refusal; hemorrhaging of tissue in intestine, liver, stomach and bladder; uterine enlargement; and death.

Table II. Color reaction of various trichothecene standards.

Trichothecene	Color Reaction		
	[a]NBP/TEPA	[b]*p*-Anisaldehyde	[c]Sulfuric acid
T-2 toxin	Blue	Purple	Gray
Dicetoxyscirpenol	Blue	Purple	Purple
3-Acetoxynivalenol	Blue	Carnary yellow	Brown
Fusarenone-X	Blue	Carnary yellow	Brown
Monoacetoxyscirpenol	Blue	Purple	Purple
Deoxynivalenol	Blue	Carnary yellow	Brown
Nivalenol	Blue	Carnary yellow	Brown

[a]The plates were dipped in 3% w/v solution of 4-(*p*-nitrobenzyl) pyridine (NBP) in chloroform-carbon tetrachloride (2:3, vol/vol), dried and heated for 30 min at 110°C. After cooling, the plates were dipped in 10% v/v solution of tetraethylenepentamine (TEPA) in chloroform-carbon tetrachloride (2:3, vol/vol).

[b]*p*-Anisaldehyde, 0.5 m*l*, was added to the mixture of 70 m*l* methanol, 10 m*l* glacial acetic acid, and 5 m*l* conc. sulfuric acid.

[c]TLC plates were sprayed with 20% sulfuric acid in methanol and heated for 10 min at 110°C.

Three cultures out of four *F. sporotrichioides* isolates were highly toxic and all three rats per each treatment died within 3 or 4 days after feeding. Two isolates obtained from Jeongsun caused hemorrhage in stomach and intestines (Table III). Intensive hemorrhages in stomach and intestine were sometimes associated with bladder hemorrhage. One isolate obtained from Chuncheon caused severe mucosae in intestine and mild hemorrhage in bladder. No clinical sign was observed in the rats fed the culture of *F. sporotrichioides* obtained from Hongcheon.

Identification of T-2 toxin by TLC

The culture extracts for the detection of trichothecenes produced by four isolates of *F. sporotrichioides* were analyzed by TLC using three different solvent systems and made visible by three different color reagents. Table I and II illustrate the degree of resolution and color reaction of various trichothecene standards when the plates were developed in three different solvent systems.

Resolution was good when pure standards were chromatographed, but culture extracts gave considerable streaking and non-specific fluorescence which prevented identification of any trichothecenes when spraying with *p*-anisaldehyde and with 20% sulfuric acid. However NBP-TEPA color reagents gave more specificity for diagnosis of trichothecenes than sulfuric acid and *p*-anisaldehyde. Few of lipids and pigments present in the culture extracts were known to react with NBP-TEPA reagents, hence the trichothecenes could be detected due to lesser background interference (Takitani *et al.*, 1979).

By this method, T-2 toxin was produced by two isolates out of four *F. sporotrichioides* isolates examined. Both of T-2 producing isolates were obtained from Jeongsun. In addition three unidentified blue spots were detected with the Rf values of 0.41, 0.21, and 0.13 when the plate was developed in chloroform-methanol (9:1, vol/vol). The other two isolates did not produce any trichothecenes although one isolate obtained from Chuncheon was highly toxic to rats.

Identification of T-2 toxin and its metabolites by GC-MS

To identify T-2 toxin and its metabolites present on a TLC plate, one sample was selected and proceeded following procedure. A 1 m*l* amount of 2 m*l* of further purified extract was applied to the TLC plate and was developed in a solution of chloroform-methanol (9:1, vol/vol). The chromatograph zones at the Rf value 0.35 to 0.75 and 0.05 to 0.30 were scraped into a small flask and extracted several times with acetone, called fractions T-2-A and T-2-B, respectively. The acetone was evaporated under *vacuo*.

The two scraped fractions obtained from TLC plate were derivatized with TFA and injected into GC-MS. The gas chromatogram of

Table III. Toxicity of *Fusarium sporotrichioides* isolates to rats fed at a 1:1 mixture of fungal culture on wheat and a complete rat diet.

Location	Code number	Food consumed (g)	Wt. gain or loss (g)	Death	Toxic signs
Jeongseon	CJS-1	0.5	−20.4	+	hemorrhages in intestine, stomach, bladder
Jeongseon	CJS-2	0.6	−17.4	+	hemorrhages in intestine, stomach, bladder
Chuncheon	SCC-C2b	3.3	−15.8	+	mucosae in intestine, mild hemorrhages in bladder
Hongcheon	CSHS-E1b	38.7	+7.0	−	normal
Control	−	60.3	+14.7	−	normal

Results were based on three rats per treatment.
Control diet was a mixture of 1:1 autoclaved wheat and a complete rat diet.

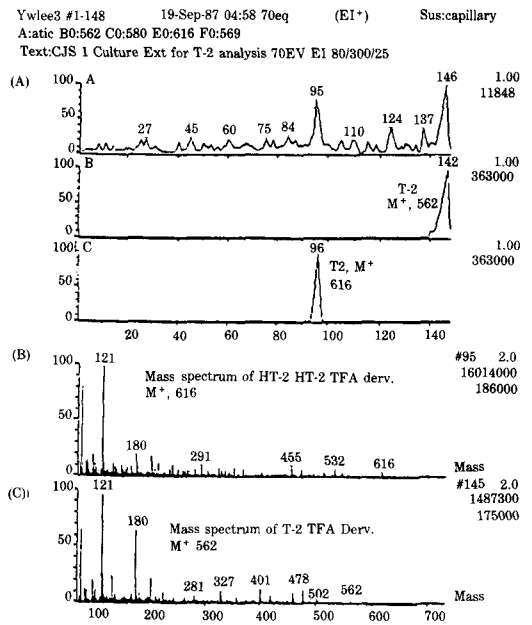


Fig. 1. Total ion current profiles of a *Fusarium sporotrichioides* culture extract with T-2 and HT-2 toxin standards (A), and full mass spectra of the trifluoroacetic (TFA) derivatives of T-2 toxin (B; scan number 95) and HT-2 toxin (C; scan number) 145).

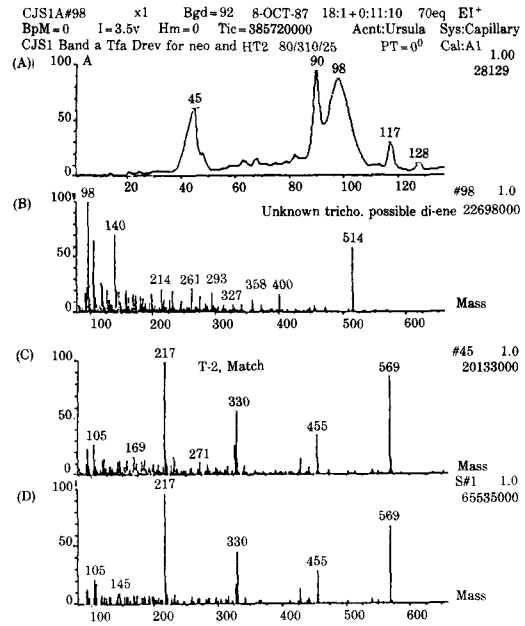
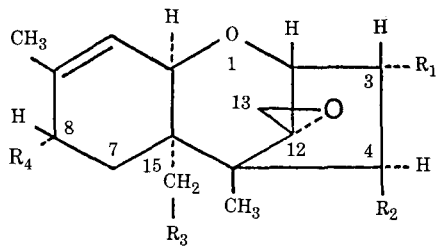


Fig. 2. Total ion current profiles of a *Fusarium sporotrichioides* culture extract (A) and full mass spectra of the trifluoroacetic (TFA) derivatives of unknown trichothecene (B; scan number 98), T-2tetraol (C; scan number 45), and T-2 tetraol standard in date base library (D).

the TFA components from fraction T-2-A is shown in Fig. 1 (A). The scans of the TFA derivatives of the peak 146 and 95 had the same retention time of T-2 TFA and HT-2 TFA, respectively. The mass spectra of TFA derivatives of two components are shown in Fig. 1 (B) and Fig. 2 (C). Mass spectra of component 146 and 95 were identical to those of T-2 TFA and HT-2 TFA standards in library data search, respectively. The gas chromatogram of the TFA components from fraction T-2-B is shown in Fig. 2 (A). The scan of the TFA derivative of the peak 45 was identified as T-2 tetraol. The mass spectrum of component 45 is exactly identical to that of T-2 tetraol in library data search (Fig. 2 (C) and Fig. 2 (D)). A mass spectrum of the TFA derivative of the peak 98 was shown in Fig. 2 (B). Fragmentation pattern of this component resembles that of a typical trichothecene, but it is still unidentified.

Therefore the metabolites of T-2 toxin shown on TLC chromatogram with the Rf values of



	R_1	R_2	R_3	R_4
a) T-2 toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$
b) HT-2 toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
c) T-2 tetraol	OH	OH	OH	OH

Fig. 3. Chemical structure of a) T-2 toxin, b) HT-2 toxin, and c) T-2 tetraol.

0.41 and 0.13 in chloroform-methanol (9:1, vol/vol) were identified as HT-2 toxin and T-2 tetraol, respectively. Another metabolite of T-2 toxin with the Rf value of 0.21 remained unknown tentatively. The chemical structures of T-2, HT-2, and T-2 tetraol are shown in Fig. 3.

T-2 toxin producing *F. sporotrichioides* was first reported by Ueno *et al.* (1972; 1973). Yagen and Joffe (1976) suggested that most of the 106 strains of *F. sporotrichioides* from the causal cereals of ATA were able to produce T-2 toxin. Scott *et al.* (1970) reported the production of T-2 toxin in six Canadian isolates of *F. sporotrichioides* . This is the first report that *Fusarium* isolates obtained from Korea produce T-2 toxin and its related trichothecenes.

摘 要

우리나라 옥수수산지로부터 분리한 *Fusarium sporotrichioides* 4균주의 밀배양체를 쥐에 경구투여하여 독성을 조사하였다. 총 4균주 중 3균주가 급여 3-4일 이내에 모든 쥐를 치사시켜 매우 높은 독성을 나타냈다. 독성 균주의 배양체를 박층크로마토그라피와 가스크로마토그라피 - 질량분석기로 분석한 결과 정선 지방에서 분리한 2균주가 T-2 독소와 관련대사물을 생성하였다. 본 보고는 *F. sporotrichioides*에 의한 T-2 독소의 생성을 우리나라에서는 처음으로 확인한 것이다.

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