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Comparative Yields of T-2 Toxin and Related Trichothecenes from Five Toxicologically Important Strains of *Fusarium sporotrichioides*

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The range and comparative yields of T-2 toxin and related trichothecenes from five toxicologically important strains of *Fusarium sporotrichioides*, i.e., NRRL 3299, NRRL 3510, M-1-1, HPB 071178-13, and F-38, were determined. Lyophilized cultures of the five strains maintained in the International Toxic *Fusarium* Reference Collection were used to inoculate autoclaved corn kernels. Corn cultures were incubated at 15°C for 21 days and analyzed for trichothecenes by thin-layer chromatography and capillary gas chromatography. All five strains produced T-2 toxin, HT-2 toxin, T-2 triol, and neosolaniol. Two strains also produced T-2 tetraol, and two others produced diacetoxyscirpenol. The highest producer of T-2 toxin (1,300 mg/kg), HT-2 toxin (200 mg/kg), T-2 triol (1.9 mg/kg), and neosolaniol (170 mg/kg) was NRRL 3510, which was originally isolated from millet associated with outbreaks of alimentary toxic aleukia in the USSR. The second highest producer of T-2 toxin (930 mg/kg) was NRRL 3299. The other three strains produced T-2 toxin at levels ranging from 130 to 660 mg/kg. Thus, the five strains differed considerably in the amounts of T-2 toxin and other trichothecenes produced under identical laboratory conditions. These strains are being maintained under optimal conditions for the preservation of *Fusarium* cultures and are available from the *Fusarium* Research Center, The Pennsylvania State University, University Park.

Many strains of *Fusarium sporotrichioides* Sherb. have been reported to produce T-2 toxin and related trichothecenes in cultures on a variety of substrates under different incubation conditions (12). Some of these strains have been deposited in the International Toxic *Fusarium* Reference Collection (ITFRC) and are being maintained in lyophilized cultures at the *Fusarium* Research Center, The Pennsylvania State University, University Park (12). Although all strains of *F. sporotrichioides* in the ITFRC have been reported to be toxic to experimental animals or to produce trichothecenes, these cultures were maintained under different storage conditions in different laboratories from the time of their original isolation until deposition in the ITFRC. Their abilities to produce trichothecenes may have deteriorated, and consequently, the actual toxin-producing potentials of the lyophilized cultures maintained in the ITFRC are unknown. The purpose of the investigation reported here was to compare the yields of trichothecenes under identical laboratory conditions by five strains of *F. sporotrichioides* (12) that have been reported to produce large amounts of T-2 toxin and have been used extensively in toxicological investigations.

MATERIALS AND METHODS

Fusarium strains. Lyophilized cultures of the five strains of *F. sporotrichioides* used in this investigation were obtained from the ITFRC.

NRRL 3299 (ITFRC T-348), as *F. tricinctum* (Corda) Sacc. strain T-2, was originally isolated from corn in France (13) and was deposited in the Northern Regional Research Laboratory, Peoria, Ill., collection as *F. tricinctum* NRRL 3299 (7). T-2 toxin was first isolated and chemically characterized from this strain by Bamburg et al. (1), and subsequently, *F. sporotrichioides* NRRL 3299 became one of the most widely used toxigenic *Fusarium* strains in the world. In addition to producing T-2 toxin, this strain is known to produce HT-2 toxin, diacetoxyscirpenol, and neosolaniol (12).

NRRL 3510 (ITFRC T-345) was originally isolated from overwintered millet associated with outbreaks of alimentary toxic aleukia of humans in the USSR and was deposited at the Northern Regional Research Center as *F. tricinctum* NRRL 3510 (6). Thus, this strain is one of only a few *Fusarium* strains originally isolated from overwintered cereals associated with alimentary toxic aleukia which is freely available to researchers on mycotoxins. This strain is known to produce T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and neosolaniol (12).

M-1-1 (ITFRC T-568), as *F. solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans. M-1-1, was originally isolated from moldy soybean hulls associated with outbreaks of bean hulls poisoning of horses in Hokkaido, Japan (10, 16). This strain is known to produce T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and neosolaniol (12).

HPB 071178-13 (ITFRC T-506) was isolated from overwintered cribbed corn in Ontario, Canada, and is known to produce T-2 toxin, HT-2 toxin, and neosolaniol (14).

F-38 (ITFRC T-249) was isolated from millet in Hungary and is known to produce T-2 toxin, HT-2 toxin, T-2 tetraol, and neosolaniol (15).

Culture techniques. A lyophilized culture of each of the strains described above was used to inoculate samples of whole yellow corn kernels (400 g of kernels and 400 ml of water) in 2-liter glass fruit jars previously autoclaved at 121°C for 1 h on each of two consecutive days. Cultures were incubated at 15°C for 21 days. In addition, cultures of *F. sporotrichioides* NRRL 3299 on corn were incubated at 25°C for 21 days, and this strain was also used to inoculate rice. Rice (1 kg) was soaked overnight in water (425 ml), dispensed into 250-ml Erlenmeyer flasks (50-g samples in each), and autoclaved at 121°C for 30 min. Cultures of this strain on rice were incubated at 15 and 25°C for 21 days. Harvested

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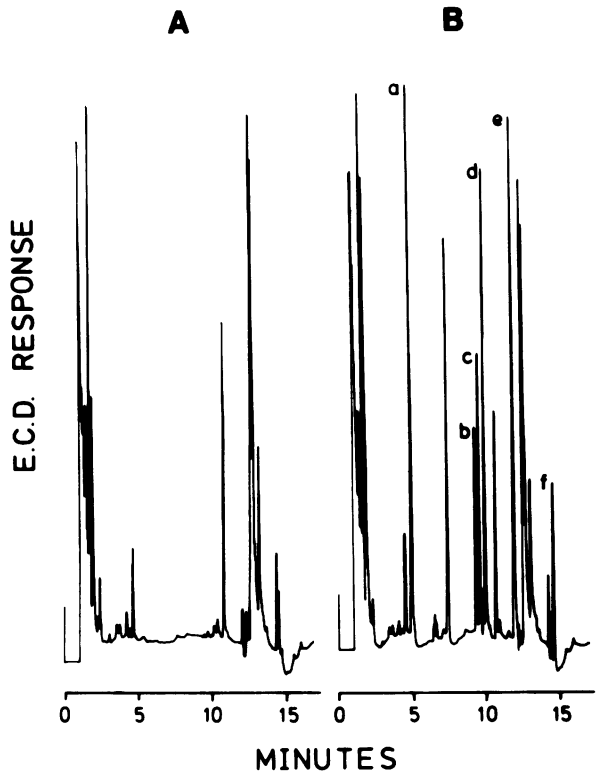

FIG. 1. Chromatograms of the first fraction (hexane-ethyl acetate [2:8]) prepared from control corn showing (A) the derivatized extract and (B) the same extract spiked with the following trichothecene standards: T-2 tetraol (a), diacetoxyscirpenol (b), neosolaniol (c), T-2 triol (d), HT-2 (e), and T-2 (f). E.C.D., Electron capture detector.

corn and rice cultures were dried in a forced-draft oven at 50°C for 24 h, milled in a Wiley mill, and stored at 5°C until used. Uninoculated corn and rice treated in the same way were used as controls.

Extraction and cleanup of corn and rice cultures. The dried and ground corn and rice cultures were extracted by adding 10 ml of distilled water to 50 g of culture material and extracting three times with 100 ml of 96% ethanol by blending for 2 min in a Waring blender and filtering. The combined filtrates were evaporated to dryness in a rotary evaporator, and the residue was made up to 50 ml in ethanol. A 10-ml sample of the ethanol extract (equivalent to 10 g of the original material) was evaporated to dryness. Between 1 and 1.5 g of Silica Gel 60 (E. Merck AG) was added to the oily residue, and the material was transferred quantitatively to the top of a column (15 mm, inside diameter) packed with 15 g of Silica Gel 60 in hexane-ethyl acetate (8:2) topped with 3 g of anhydrous sodium sulfate.

The column was washed with 200 ml of hexane-ethyl acetate (8:2), and the eluate was discarded. The trichothecenes were then eluted from the column and collected in two fractions by successive elution with 150 ml of hexane-ethyl acetate (2:8) and 150 ml of methanol-ethyl acetate (2:8). The eluates were dried under vacuum and made up to 10 ml in ethyl acetate for analysis by thin-layer chromatography and gas chromatography.

Thin-layer chromatography. Extracts of cultures were screened by thin-layer chromatography for the presence of trichothecenes, but quantification and identification were done by capillary gas chromatography. The culture extracts were chromatographed with trichothecene standards on silica gel thin-layer chromatography plates containing a fluorescence indicator (Silica Gel 60, F_{254}; E. Merck AG). The plates were developed in toluene-ethyl acetate-acetic acid (6:3:1), dried, sprayed with 20% sulfuric acid, heated for 5 min at 120°C, and viewed under UV light at 366 nm.

Gas chromatography. Quantitative determination of the trichothecenes was performed by capillary gas chromatography of derivatized extracts on a gas chromatograph (model 5300; Carlo Erba) equipped with an electron capture detector and split injection.

Extracts (1 ml, equivalent to 1 g of the original material) were evaporated to dryness, dissolved in 1 ml of toluene-acetonitrile (95:5), and heated for 1 h at 60°C with 100 μl of *n*-heptafluorobutyrylimidazole. After being cooled, 1 ml of 0.1 M sodium phosphate buffer (pH 6.0) was added to each derivatization vial, and the contents were mixed thoroughly. The phases were allowed to separate, and a 25-μl sample of the organic phase was placed in a separate vial, evaporated to dryness, dissolved in 1 ml of benzene, and analyzed by gas chromatography after being suitably diluted.

Chromatographic conditions. The derivatized extracts were separated on a fused-silica capillary column (25 m by 0.32 mm, inside diameter) coated with a film of SE-30 (0.25 μm thick; Macherey-Nagel) under the following chromatographic conditions: carrier gas (helium) flow rate, 34 cm/s; detector makeup gas (nitrogen) flow rate, 30 ml/min; injection volume, 1 μl; injector split ratio, 10:1; injector temperature, 200°C; detector temperature, 300°C; oven temperature profile of 180 to 220°C at 4°C/min, 220 to 250°C at 15°C/min and then 250°C maintained for 5 min.

The chromatograms were screened for the presence of T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol, and diacetoxyscirpenol by comparison with a chromatogram of a mixture of these standards and by chromatographic analysis of extracts spiked with a mixture of the standards (Fig. 1). Quantitative determinations of individual toxins were done by comparison of peak heights and calibration curves constructed for each individual trichothecene over the concentration range of 15 to 400 pg/μl of toxin injected. Trichothecene standards used in this study were isolated and characterized by elemental analysis, optical rotation data, and infrared, nuclear magnetic resonance, and mass spectra as described by Yagen et al. (20). The two purified extracts obtained from each culture were analyzed. In general, T-2 toxin and diacetoxyscirpenol eluted only in the first-collected fraction from the silica cleanup column, and T-2 tetraol and T-2 triol appeared only in the second fraction, while neosolaniol and HT-2 toxin occurred in both. When a specific trichothecene was detected in both extracts, the values were added together.

RESULTS AND DISCUSSION

The chromatographic analysis of extracts of control corn revealed that this corn contained none of the trichothecenes tested for (Fig. 1A). The chromatographic resolution obtained between individual trichothecenes and interfering substances in extracts of control corn spiked with trichothecene standards is shown in Fig. 1B. Recoveries of T-2 toxin from control corn and rice spiked before extraction were 74 and 81%, respectively.

The yields of trichothecenes by *F. sporotrichioides* NRRL 3299 in cultures on corn and rice incubated at 15 and 25°C (Table 1) were determined in a preliminary experiment to

TABLE 1. Effects of substrate and incubation temperature on the yield of trichothecenes from *F. sporotrichioides* NRRL 3299

Substrate and incubation temp (°C)	Trichothecene yield (mg/kg)[a]					
	T-2 toxin	HT-2 toxin	T-2 triol	T-2 tetraol	Neosolaniol	Diacetoxyscirpenol
Corn						
15	840	120	1.2	ND	110	2.6
25	160	69	1.7	28	58	ND
Rice						
15	250	26	0.8	0.4	48	ND
25	100	32	1.5	18	270	ND

[a] ND, Not detected.

establish the most suitable substrate and temperature for comparison of the five strains. NRRL 3299 produced larger amounts of T-2 toxin and other trichothecenes (except neosolaniol) on corn than on rice, and on both substrates, higher yields of T-2 toxin were obtained at 15 than at 25°C. The finding that strain NRRL 3299 produced more T-2 toxin on corn than on rice differs from some previous reports on T-2 toxin production by this strain on these substrates (2, 3) but agrees with the results of Cullen et al. (8). In the study reported here, maximal yields of T-2 toxin (840 mg/kg) from this strain were obtained in cultures on corn incubated at 15°C (Table 1). Consequently, it was decided to compare the yields of T-2 toxin and other trichothecenes from five strains of *F. sporotrichioides* cultured on corn at 15°C, although less purification is needed to remove interfering substances from rice cultures than from corn cultures (2).

The yields of trichothecenes from five strains of *F. sporotrichioides* cultured on corn incubated at 15°C for 21 days are given in Table 2. All of the strains produced T-2 toxin, HT-2 toxin, T-2 triol, and neosolaniol; two strains also produced T-2 tetraol; and two others produced diacetoxyscirpenol.

The highest level of T-2 toxin (1,300 mg/kg), as well as of HT-2 toxin, T-2 triol, and neosolaniol, was produced by *F. sporotrichioides* NRRL 3510 (Table 2). This strain has previously been reported to produce unspecified amounts of T-2 toxin, together with HT-2 toxin, diacetoxyscirpenol, and neosolaniol (5, 6, 7, 17–19). It is interesting that strain NRRL 3510, which was isolated from overwintered millet associated with alimentary toxic aleukia in the USSR (6), was found to be the highest producer of T-2 toxin among the strains compared in this study. Strains of *F. sporotrichioides* from overwintered cereals associated with alimentary toxic aleukia have previously been reported to produce much higher levels of T-2 toxin than strains from other sources (11).

TABLE 2. Yields of trichothecenes from strains of *F. sporotrichioides* in cultures on corn incubated at 15°C for 21 days

Strain no.	Trichothecene yield (mg/kg)[a]					
	T-2 toxin	HT-2 toxin	T-2 triol	T-2 tetraol	Neosolaniol	Diacetoxyscirpenol
NRRL 3299	930	110	1.6	ND	87	2.8
NRRL 3510	1,300	200	1.9	1.1	170	ND
M-1-1	660	120	1.3	ND	120	1.3
HPB 071178-13	460	64	1.1	ND	100	ND
F-38	130	36	1.7	7.0	41	ND

[a] ND, Not detected.

The second-highest level of T-2 toxin (930 mg/kg) was produced by *F. sporotrichioides* NRRL 3299, which also produced HT-2 toxin, T-2 triol, neosolaniol, and diacetoxyscirpenol (Table 2). The production of T-2 toxin by this strain, usually referred to as *F. tricinctum* T-2 or *F. tricinctum* NRRL 3299, has been reported for a variety of media, and this strain has been used extensively for the production of T-2 toxin for toxicological investigations (12). High yields of T-2 toxin by this strain in cultures on corn incubated under various temperature regimes have been reported, e.g., 1,515 mg/kg reported by Ikediobi et al. in 1971 (9) and 8,300 mg/kg reported by Burmeister in 1971 (4). Yields reported in more recent publications are usually much lower, e.g., 500 mg/kg reported by Bottalico et al. in 1983 (3) and 125 mg/kg reported by Bata et al. in 1984 (2), although in 1982, Cullen et al. (8) reported a yield of 2,700 mg/kg. These findings suggest that the subcultures of *F. sporotrichioides* NRRL 3299 that are in use in various laboratories have deteriorated to different degrees in their ability to produce T-2 toxin. However, the different yields of T-2 toxin reported for this strain in cultures on corn may also be due to differences in incubation conditions and analytical methods. In our laboratory, lyophilized ITFRC cultures of this strain produced almost identical yields of trichothecenes under standardized conditions in different experiments (Tables 1 and 2).

The other three strains used in this study produced T-2 toxin at levels ranging from 130 to 660 mg/kg, and strain M-1-1 was the second highest producer of HT-2 toxin and neosolaniol (Table 2).

Our investigation has established that lyophilized cultures of five toxicologically important strains of *F. sporotrichioides* deposited in the ITFRC still have the ability to produce T-2 toxin and several related trichothecenes in cultures on corn incubated at 15°C for 21 days. The five strains differed considerably in the amounts of T-2 toxin and other trichothecenes produced under identical laboratory conditions. These strains are being maintained under optimal conditions for the preservation of *Fusarium* cultures (12) and are available from the *Fusarium* Research Center.

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