

Correlation of Biological to Chromatographic Data for Two Mycotoxins Elaborated by *Fusarium*¹

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Thirty-seven identified strains of *Fusarium*, most of them isolated from fescue grass, were tested for their ability to elaborate mycotoxins in laboratory culture. The presence of the toxins was determined by infrared light, thin-layer chromatography, mouse toxicity, fungistatic effects, and phytotoxic properties. A good correlation was demonstrated between T-2 toxin detection by thin-layer chromatography and inhibition of *Rhodotorula rubra* by culture extracts. All of the strains producing either butenolide or T-2 toxin were toxic to mice with but one exception; those producing T-2 toxin inhibited growth of the yeast.

The possibility that fescue foot, a disease occasionally seen in cattle grazing tall fescue (*Festuca arundinacea* Schreb.), could be caused by a fungus growing on the grass has been considered for some time (2, 5). Two mycotoxins, 4-acetamido-4-hydroxy-2-butenolide (butenolide) and 4 β -15-diacetoxy-8 α -(3-methylbutyryloxy)-12, 13-epoxytrichothec-9-en-3 α -ol (T-2 toxin), produced by *Fusarium tricinctum* NRRL-3249 have been characterized (4).

Additional work on mold isolates from toxic tall fescue and nearby tall fescue or orchard grass (*Dactylis glomerata* L.) pastures has shown that almost all of the toxin-producing isolates examined belong to the genus *Fusarium* (5). The toxicity to mice of extracts of 28 or 29 of these isolates grown on Sabouraud agar was due to either the butenolide, the T-2 toxin, or both (3). The effects of culture extracts of the *Fusarium* strains on pea seed germination and on a yeast strain, *Rhodotorula rubra* Y-7222, sensitive to T-2 toxin are compared in this paper with previously reported mouse toxicity and chemical data (3).

White corn grits (WCG) fermented with *F. tricinctum* NRRL 3299 yielded several grams of this mycotoxin per kilogram of substrate (H. R. Burmeister and C. W. Hesseltine, *Bacteriol. Proc.*, p. 14, 1970). A combination of cultural conditions that promote high yields of T-2 toxin and of a sensitive microbiological indicator enabled us to evaluate further the toxin-producing potential of *Fusarium* strains isolated from fescue grass.

MATERIALS AND METHODS

Origin of cultures. Strain NRRL 3249 was an early isolate from toxic tall fescue hay from Missouri (2). Strain NRRL 3299 was given to us by E. B. Smalley, Univ. of Wisconsin, as his T-2 strain isolated from toxic corn. The other isolates used in this study were taken from six samples of tall fescue gathered in a pasture where 11 out of 100 cattle were severely affected with fescue foot; two samples of tall fescue and one sample of orchard grass were gathered nearby (3). The isolates consisted of four species of *Fusarium*. Five of seven strains identified as *F. lateritium* produced the *Gibberella* stage under appropriate conditions.

Preparation of culture extracts. Each isolate was grown on 30 g of WCG moistened with 15 ml of water and on 20 ml of Sabouraud agar. Cultures were incubated at 15 C for 21 days. Extracts of the agar cultures were prepared and treated as described previously (3). For fungistatic effects and phytotoxic properties, duplicate samples of each fermented medium were extracted with 100 ml of chloroform-acetone (85:15) by blending in a Waring Blendor with the solvents for 3 to 4 min. Fifty milliliters of the solvent was recovered from the substrate by filtering through paper toweling. The extracts were evaporated at room temperature.

Those cultures re-examined (culture no. 2, 5, 8, 28, 29, and 34) for toxicity to the mouse and for the presence of butenolide and T-2 toxin by the methods previously described (3) were removed after 38 days. Extracts of these six cultures grown on WCG were treated similarly to the cultures grown on Sabouraud agar, but modifications were necessary since much more inert material is extracted from WCG. Infrared (IR) spectra of the extract residues in a 1-ml cell (4 mg of residue from Sabouraud agar cultures/ml of CH₂Cl₂; 8 mg of residue from WCG/ml of CH₂Cl₂) showed the presence of butenolide (absorption at

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1,705, 1,760, and 1,790 cm^{-1}) in culture extracts from strains 28 and 34 although none was detected by thin-layer chromatography (TLC).

Biological tests for mycotoxins. Fungistatic effects of the culture extracts were determined with a yeast, *R. rubra* NRRL Y-7222. After the solvents were evaporated, the extract residue was mixed with 5 ml of acetone. An antibiotic assay disc (12.7 mm; Schleicher & Schuell no. 740E) was saturated with the acetone-extract residue, dried, and placed on the surface of yeast-malt (YM) agar inoculated with cells of Y-7222. The indicator plates were prepared by adding 0.1 ml of the yeast culture (the cells were diluted in YM broth to give a reading of 50% transmittance at a wavelength setting of 600 nm) to 6 ml of YM agar. The inoculated agar was poured into a standard petri dish.

Pea seeds, *Pisum sativum*, soaked in water containing 2 μg of T-2 toxin per ml failed to germinate, even though they were not affected by as much as 200 μg of butenolide per ml (1). A wrinkled seed variety

of pea, Little Marvel, was used in this study. Ten seeds, surface sterilized in a 0.1% solution of mercuric chloride for 5 min, were soaked for about 16 hr in an extract residue suspended in 25 ml of water. The turgid seeds were placed between moistened filter paper and incubated at 28 C. After a 4-day incubation period, the germinated seeds were counted. A single germinated seed indicated the lack of T-2 toxin or its presence at a level below 2 $\mu\text{g}/\text{ml}$.

RESULTS AND DISCUSSION

Results of the biological tests for toxic products produced by strains of *Fusarium* and qualitative chemical tests for butenolide and T-2 toxin are presented in Table 1. Since most of the chemical and mouse toxicity results were reported in an earlier paper (3), those data were used to evaluate the other biological indicators of toxicity, i.e., inhibition of *R. rubra* and in-

TABLE 1. Biological indicators and chromatographic evidence for the presence of two mycotoxins produced by four *Fusarium* species

Species	Strain no. ^a	Toxicity indicator			Toxin	
		Mouse	Pea seed germination	<i>Rhodotorula rubra</i>	T-2 ^b	Butenolide ^b
<i>F. tricinctum</i>	NRRL 3249	+	+	+	+	-
	12, 19	+	+	+	+	-
	NRRL 3299	+	+	+	+	+
	3, 9-11, 13, 34 ^{d, e}	+	+	+	+	+
	2 ^d	-	+	-	-	-
	8 ^d	-	-	-	-	-
	33	-	-	-	ND	ND
	15	+	?	+	?	+
<i>F. semitectum</i>	1, 4	+	+	+	+	+
	35-40	-	-	-	ND	ND
<i>F. equiseti</i>	5 ^d	-	-	-	-	-
	6	+	+	+	+	-
	16	+	-	-	+	+
	18, 20	+	+	+	+	+
	21, 22	+	-	+	+	+
	41	-	-	-	ND	ND
	17 ^f	+	+	+	+	+
<i>F. lateritium</i>	25 ^f , 26 ^f	+	+	-	-	+
	24 ^f	-	-	-	-	-
	28 ^{d, e, f}	+	+	+	-	+
	29 ^d	+	+	-	-	-
	42	-	-	-	ND	ND

^a Strain numbers 1 through 32 are as reported in reference 3.

^b T-2 = 4 β ,15-diacetoxy-8 α -(3-methylbutyryloxy)-12,13-epoxytrichothec-9-en-3 α -ol; thin-layer chromatographic (TLC) evidence: detection limit of the pure compound is of the order of 10 μg . Butenolide = 4-acetamido-4-hydroxy-2-butenic acid γ -lactone; TLC evidence: detection limit of the pure compound is of the order of 10 μg . Infrared (IR) evidence, detected in CH_2Cl_2 solutions containing 0.1 or more mg/ml.

^c + = Death or inhibition; LD₅₀ of the butenolide in mice is 43.6 mg/kg (ip) and of the T-2 toxin in mice is 3.0 mg/kg (ip; reference 4); - = no adverse response; ND = not determined.

^d Results of re-examination of these strains varied from those previously reported.

^e Butenolide detected only in Sabouraud agar culture by IR.

^f *Gibberella* stage observed.

hibition of pea seed germination. For strains 2, 5, 8, 28, and 29, the present data did not agree with those previously reported (3); these strains were re-examined for mouse toxicity and the presence of butenolide or T-2 toxin. These new data are entered in Table 1. Earlier, strains 2 and 8 were toxic to mice and T-2 toxin was detected, but now these strains are nontoxic. TLC and IR indicated that neither T-2 toxin nor butenolide was produced. In the earlier report (3), strains 28 and 29 were questionably toxic to mice. When these strains were cultured on WCG, they were toxic although neither butenolide nor T-2 toxin was detected. Previously, strain 5 was questionably toxic to mice and produced T-2 toxin and a trace of butenolide; however, on WCG it was not toxic to mice, and neither butenolide nor T-2 toxin was detected.

Pea seed germination and growth of the yeast are both inhibited by microgram quantities of T-2 toxin but are not affected by relatively large quantities of butenolide (1). Therefore, the pea seed and yeast tests were used to indicate the presence of T-2 toxin or of unidentified phytotoxins or fungistatic agents produced by these *Fusarium* strains.

The yeast test is the preferred biological indicator of T-2 toxin since *R. rubra* is inhibited by assay discs containing 4 μg of toxin (1). Results are available within 24 hr, the method can be made semiquantitative, and it is more specific than animal tests. Production of T-2 toxin by 20 of the 37 strains was indicated by TLC, and extracts from all but one of these, strain 16, inhibited *R. rubra*. Even though extracts from strain 28 contained no T-2 toxin as indicated by TLC, still they inhibited the yeast. This fungistatic effect could be caused by the presence of T-2 toxin not detected by TLC or by other fungistatic agents. Pea germination tests were difficult to interpret because the seeds, even though surface-sterilized, frequently were attacked by bacteria before they were able to germinate, and a germination ratio of 1 of 10

compared to 10 of 10 seeds did not indicate an increase in toxin but rather indicated seeds contaminated by microorganisms or factors that we were unable to control.

Extracts toxic to mice obtained from strains of *F. tricinctum*, *F. semitectum*, and *F. equiseti* contained either butenolide or T-2 toxin, but one strain of *F. lateritium*, 29, was toxic to mice in the absence of detectable amounts of butenolide or T-2 toxin.

The species *F. tricinctum* and *F. equiseti* contained strains that produced no toxin, both toxins, or only one of the toxins. The two strains of *F. semitectum* which were examined produced both toxins. Only one of the *F. lateritium* isolates produced T-2 toxin, butenolide was produced by four of seven strains, and one of the strains was toxic to mice but did not produce either T-2 toxin or butenolide (Table 1).

We conclude that the inhibition of *R. rubra* is a good indicator for the presence of T-2 toxin in culture extracts of *Fusarium* isolates. In this study, a good correlation was demonstrated between the inhibition of the yeast and the detection of T-2 toxin by TLC in culture extracts. Pea seed germination, although a sensitive T-2 toxin indicator when seeds imbibe toxin from a sterile solution, was not a reliable assay for culture extracts because the extracts are easily contaminated and allow growth of microorganisms that attack the seeds.

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