Biological Assays for Two Mycotoxins Produced by *Fusarium tricinctum*

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A survey was made to detect microorganisms useful for assaying butenolide [4-acetamido-4-hydroxy-2-butenoic acid γ-lactone] and T-2 toxin [4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytricothec-9-en-3α-ol]. These mycotoxins produced by strains of *Fusarium tricinctum* have been implicated in mycotoxicosis of livestock. Although butenolide proved to be a very weak antibiotic, assay discs containing 100 μg of this toxin inhibited *Spirillum serpens* NRRL B-2052, *Vibrio tyrogenes* NRRL B-1033, and *Xanthomonas campestris* NRRL B-1459. T-2 toxin had no effect on 54 bacterial strains but inhibited 6 of 11 fungi. Growth of *Rhodotorula rubra* NRRL Y-7222 and *Penicillium digitatum* NRRL 1202 was retarded by assay discs containing 4 μg of T-2 toxin. Solutions with less than 1 μg of T-2 per ml toxin were readily detected by a pea seed germination test. Germination was reduced more than 50% when seeds imibed solutions of 0.5 μg of T-2 toxin per ml. Butenolide had no effect on pea seed germination at concentrations as high as 200 μg/ml.

Toxic feeds are being associated more and more with specific fungi that cause certain mycotoxicoses in farm animals. Identification, production, purification, and biological testing of fungal metabolites suspected of being toxic have aided in elucidating the cause of many feeding problems.

Mycotoxins toxic to animals are elaborated by strains of *Fusarium tricinctum* isolated from fescue, *Festuca arundinacea*, a valuable forage grass used as winter pasture in the Southern and Western areas of the United States. These isolates have been implicated in a disease known as fescue foot (6–9). Yates et al. (8) characterized a toxic butenolide, 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone, from these strains cultured on Sabouraud agar at low temperature. Reportedly, *F. tricinctum* was the fungus most prominent on moldy corn, and strains of this species produced the most potent toxins of the molds isolated from toxic corn (5). Bamberg et al. (1) tested culture filtrates of hundreds of molds taken from moldy grain; the filtrates most toxic to mice were obtained from strains of *F. tricinctum*. Among the several mycotoxins elaborated by this fungal species, the formation of the major toxic metabolite depends upon cultural conditions and on the particular fungal strain. In laboratory culture, strains isolated from moldy grain yield diacetoxyscirpenol, T-2 toxin, and 4-desacetoxy T-2 toxin (3; J. R. Bamberg, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1969).

The T-2 toxin characterized as 4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytricothec-9-en-3α-ol (2) has an LD₅₀ of 4 mg/kg of body weight when administered orally to mice (1). The butenolide has an LD₅₀ of 275 mg/kg of body weight when given orally to mice and 44 mg/kg when injected into the peritoneum.

An application of the culture filtrates to the skin of laboratory animals provides the simplest test for these mycotoxins (6; J. R. Bamberg, Ph.D. Thesis; E. B. Smallkey et al., Proc. 1st U. S.-Jap. Conf. Toxic Microorganisms, *in press*). Toxicity is indicated by an edematous, hemorrhagic skin response, and the severity of the response is graded to aid in estimating toxin quantities (J. R. Bamberg, Ph.D. Thesis). Although thin-layer chromatography is useful in detecting T-2 toxin in partially purified extracts, small amounts of toxin recovered along with impurities are difficult to quantitate. Because of the difficulties in detecting low levels of T-2 toxin by thin-layer or paper chromatography, Bamberg (Ph.D. Thesis) developed a gas-liquid chromatographic (GLC) procedure for measuring the trimethylsilyl ether of the toxin in extracts of mold cultures and corn samples. As for T-2 toxin, a simple chemical method for quantitating butenolide in fungal extracts has not been developed; however, its presence can be detected and its relative amount estimated by infrared spectroscopy (9).

Current chemical and biological tests for de-
testing butenolide or T-2 toxin are time-consum ing, require expensive equipment, and with the possible exception of the GLC procedure, are only crude estimates of the toxin quantities in culture extracts. The animal skin test has proved the most useful thus far in confirming the potency of culture filtrates. Since animals were sensitive to these toxins, some other cellular system should also react to their presence. If a simple test could be developed for detecting these mycotoxins, it could aid in evaluating the toxic potential of moldy grains. Various microorganisms are used to estimate the quantity of growth inhibitors in crude extracts of many products. Because toxin-sensitive microorganisms provide a quick and easy method of estimating toxin quantities, a survey was carried out to find bacteria and fungi inhibited by T-2 toxin or butenolide. In addition to microorganisms, a pea seed germination test was made because diacetoxyscirpenol, a compound with a molecular structure similar to T-2 toxin, inhibited pea stem elongation in solutions of 1 μg/ml (4).

Both butenolide and T-2 toxin reportedly have antibiotic properties. At concentrations of 10 mg/ml, the butenolide inhibited 11 of 14 bacteria and 2 of 3 molds that were studied. Only slight activity was detected at 1 mg/ml and none at 0.1 mg/ml (9). Even though T-2 toxin was said to have weak fungistatic properties (J. R. Bambug, Ph.D. Thesis), a definitive method and identification of test fungi were not published in support of this finding.

MATERIALS AND METHODS

Survey for sensitive microorganisms. Acetone solutions of crystalline butenolide or T-2 toxin were added to antibiotic assay discs (no. 740E Schleicher and Schuell Co.) to give discs containing 50 μg of T-2 toxin or 200 μg of butenolide. Acetone was removed by warming the discs for 30 min at 70 C. The mycotoxins were obtained from our Industrial Crops Laboratory, the butenolide having been chemically synthesized and the T-2 toxin having been extracted from mold cultures.

Bacteria and fungi from the Agricultural Research Service Culture Collection were selected for testing. Microbes were transferred into a suitable medium and incubated at temperatures permitting good growth. The bacteria were generally propagated in nutrient broth at 32 C, and the fungi were propagated on yeast-malt (YM) agar at 22 to 27 C. After an incubation of 24 to 36 hr, 0.1 ml of bacterial suspension was spread over the surface of 20 ml of nutrient agar medium in a standard petri dish. The surface was allowed to dry for about 1 hr before the toxic filter discs were placed onto the surface. Heavy suspensions of 48-hr yeast cells and mold conidia were prepared in water. The fungal growth was loosened with a loop and suspended by vibrating before the 0.1 ml of cell suspension was spread onto the surface of YM agar. After a suitable incubation time, inhibition zones of sensitive microorganisms were measured. Zones recorded as + had a diameter of less than 16 mm; those recorded as ++ had greater diameters.

Standardized assay with sensitive microorganisms. Microorganisms indicating the greatest sensitivity to either mycotoxin were selected for testing by a standardized procedure. Rhodotorula rubra NRRL Y-722 was used to quantitate T-2 toxin, and Spirillum serpens NRRL B-2052, Vibrio tyrogenes NRRL B-1033, and Xanthomonas campestris NRRL B-1459 served as indicators of butenolide concentrations.

Slant cultures of the selected microorganisms were grown on YM agar. R. rubra was incubated for 48 hr and the bacteria were incubated for 24 hr at 32 C before assay plates were prepared. Cell suspensions were prepared by washing incubated slants and diluting the cells in YM broth to give an optical density (measured in a Spectronic-20 spectrophotometer at 600 nm) of 0.15 for the bacteria and 0.3 for R. rubra. The standardized inoculum, 0.1 ml, was added to 5 ml of melted sterile YM agar cooled to 45 C. Inoculated agar was poured into a standard 100X, 15-mm petri dish. Warming the dishes to 50 C before pouring kept the agar from rapidly solidifying and enabled the inoculated agar to spread.

Known quantities of the mycotoxins were dissolved in acetone and added by pipette to the filter paper discs (0, 25, 50, 100, and 200 μg of butenolide and 0, 2, 4, 6, 8, and 10 μg of T-2 toxin). Before being placed on the inoculated medium, the mycotoxic discs were dried at 70 C for 30 min and the zones of inhibition were measured with a ruler after incubation for 44 to 48 hr.

Pea seed germination test. Little Marvel, a wrinkled-seed variety of Pisum sativum, was tested for germination after inhibition of mycotoxin solutions. A measured quantity of crystalline T-2 toxin was dissolved in acetone and a slant line was transferred to a 100-ml beaker. After the acetone evaporated, 50 ml of water was poured into the beaker and the small quantity of toxin dissolved in it. The butenolide was diluted with water. Duplicate tests were done by steeping 25 pea seeds overnight in the toxic solutions; the turgid seeds were then planted in wet sand and covered with moist paper toweling. After 4 days of incubation at 22 to 27 C, the seeds that had germinated were counted.

RESULTS

The antibiotic activity of T-2 toxin and butenolide against selected microorganisms is presented in Table 1. Butenolide concentrations of 200 μg per assay disc inhibited 9 of 54 bacteria but none of the fungi. S. serpens NRRL B-2052, V. tyrogenes NRRL B-1033, and X. campestris NRRL B-1459, as indicated by the diameter of the inhibition zone, were more sensitive to butenolide than the other bacteria. In a standardized assay, 50 μg of butenolide noticeably inhibited each
TABLE 1. Microorganisms surveyed for butenolide and T-2 toxin sensitivity

| Microorganism                  | No. of strains or species tested | Mycotoxin\(^a\) | Butenolide 200 μg/assay disc | T-2 toxin 50 μg/assay disc |
|--------------------------------|----------------------------------|-----------------|------------------------------|--------------------------|
| Bacteria                       |                                  |                 |                              |                          |
| Rhodospirillum rubrum           | 1                                | +               | -                            |                          |
| Spirillum serpens               | 1                                | +               | -                            |                          |
| Xanthomonas campestris          | 1                                | +               | -                            |                          |
| X. campestris                   | 5                                | +               | -                            |                          |
| Vibrio tyrogenus                | 2                                | +               | -                            |                          |
| Vibrio sp.                      | 5                                | +               | -                            |                          |
| Pseudomonas denitrificans       | 1                                | +               | -                            |                          |
| Pseudomonas sp.                 | 8                                | -               | -                            |                          |
| Photobacterium Fischeri\(^b\)  | 1                                | +               | -                            |                          |
| Aeromonas liquefaciens          | 1                                | -               | -                            |                          |
| Zoogloea ramigera               | 1                                | -               | -                            |                          |
| Alcaligenes faecalis            | 1                                | -               | -                            |                          |
| Acrobacter sp.                  | 2                                | -               | -                            |                          |
| Flavobacterium sp.              | 2                                | -               | -                            |                          |
| Enterobacteriaceae (5 genera)   |                                  |                 |                              |                          |
| Micrococcaceae (3 genera)       | 6                                | -               | -                            |                          |
| Brevibacterium divaricatum      | 1                                | -               | -                            |                          |
| Cellulomonas biazotea           | 1                                | -               | -                            |                          |
| C. uda                         | 1                                | -               | -                            |                          |
| Bacillus sp.                    | 4                                | -               | -                            |                          |
| Mycobacterium phlei            | 1                                | -               | -                            |                          |
| Fungi                           |                                  |                 |                              |                          |
| Rhodotorula rubra               | 1                                | -               | +                            |                          |
| R. glutinis                     | 1                                | -               | +                            |                          |
| Saccharomyces carlsbergensis    | 1                                | -               | +                            |                          |
| S. pastorianus                  | 1                                | -               | +                            |                          |
| Candida krusei                  | 1                                | -               | +                            |                          |
| C. albicans.                   | 1                                | -               | +                            |                          |
| Cryptococcus albidos            | 1                                | -               | +                            |                          |
| Aureobasidium pullulans         | 1                                | -               | +                            |                          |
| Tremella mesenterica            | 1                                | -               | +                            |                          |
| Penicillium digitatum           | 1                                | -               | +                            |                          |
| Mucor ramannianus               | 1                                | -               | +                            |                          |

\(^a\) Symbols: +, inhibition; -, no inhibition.
\(^b\) Tested on glucose-tryptone medium plus 2% NaCl.

T-2 toxin was not bacteriostatic at a concentration of 50 μg per disc but was toxic to 6 of 11 fungi (Table 1). R. rubra NRRL Y-7222 and Penicillium digitatum NRRL 1202 exhibited larger inhibition zones than the other fungi. Discs with 4 μg of T-2 toxin caused a detectable fungistatic activity against R. rubra, and zone diameters of more than 20 mm around discs with 8 and 10 μg of toxin were recorded (Table 2).

Low levels of T-2 toxin reduced the germination percentage of pea seeds (0 μg/ml, germination percentage = 94%; 0.25 μg/ml, 90%; 0.5 μg/ml, 48%; 1.0 μg/ml, 36%; 2.0 μg/ml, 10%; 3.0 μg/ml, 0%; 4.0 μg/ml, 0%); whereas germination was not noticeably reduced by relatively large amounts of butenolide (0 μg/ml, germination percentage = 96%; 100 μg/ml, 82%; 200 μg/ml, 88%). Imbibed solutions containing 1 and 2 μg of T-2 toxin reduced the germination percentage to 36 and 10%, respectively.

**DISCUSSION**

Neither the microorganisms nor the pea seeds provide an indicator for measuring low levels of butenolide. A standard 12.7-mm filter disc with 0.1 ml of toxic filtrate would not inhibit any of the indicator bacteria if the filtrate contained less than 0.5 mg of butenolide per ml. Despite their tolerance to this mycotoxin, S. serpens, V. tyro- genus, and X. campestris could be useful in studying the effects of butenolide in a living system.

In contrast to butenolide, T-2 toxin was fungistatic and phytotoxic but was not active against bacteria. T-2 toxin was fungistatic at concentrations comparable to those causing an inflammatory skin response of animals receiving topical doses. Toxic solutions containing more than 40 μg/ml were detectable with R. rubra as the indicator fungus. Despite our emphasis on finding toxin-sensitive microorganisms, the biological test most easily standardized and requiring the lowest concentration of T-2 toxin was the pea germina-

**TABLE 2. Inhibition of test microbes by butenolide and T-2 toxin**

| Microorganism                  | Butenolide (μg/assay disc) | T-2 toxin (μg/assay disc) |
|--------------------------------|----------------------------|--------------------------|
|                               | 25 | 50 | 100 | 200 | 0  | 2  | 4  | 8  | 10 |
| Spirillum serpens NRRL B-2052  | 0  | 14 | 16  | 24  | 0  | 0  | 0  | 0  | 0  |
| Xanthomonas campestris NRRL B-1459 | 0  | 14 | 16  | 22  | 0  | 0  | 0  | 0  | 0  |
| Vibrio tyrogenus NRRL B-1033    | 0  | 14 | 16  | 20  | 0  | 0  | 0  | 0  | 0  |
| Rhodotorula rubra NRRL Y-7222   | 0  | 0  | 0   | 0   | 0  | 0  | 16 | 20 | 22 |

\(^a\) Diameter of zones of inhibition measured in millimeters.
\(^b\) Zones of inhibition are hazy.
tion test. Germination was inhibited by 50, 64, and 90% when seeds were soaked overnight in solutions containing only 0.5, 1.0, and 2.0 µg of T-2 toxin per ml, respectively.

The effect of T-2 toxin on biological agents suggest that a combination of animal, plant, and microbial test may provide a simple, although presumptive, means of estimating its quantity in culture filtrates or extracts of feeds molded with *F. tricinctum*.

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