Zearalenone Production by *Fusarium* Species

RODNEY W. CALDWELL, JOHN TUIE, MARTIN STOB, AND ROBERT BALDWIN

Department of Botany and Plant Pathology and Department of Animal Science, Purdue University, Lafayette, Indiana 47907

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One-hundred-and-thirteen isolates of *Fusarium* were tested for their ability to produce zearalenone on autoclaved corn. They belonged to the following species (number of producers per number tested): *F. episphaeria*, (0/1); *F. moniliforme*, (0/8); *Gibberella fujikuroi*, (0/3); *F. nivale*, (0/7); *F. oxysporum*, (0/15); *F. roseum*, (31/51); *F. solani*, (0/9); *F. tricinctum* (3/19). The isolates of individual species produced the following amounts of zearalenone per gram of corn: 3 isolates of *F. roseum* (0.6 to 119 μg), 3 of *F. roseum* "Culmorum" (1 to 210 μg), 3 of *F. roseum* "Equisetii" (0.6 to 2.0 μg), *F. roseum* "Gibbosum" (115 to 175 μg), 21 of *F. roseum* "Graminearum" (0.2 to 230 μg), and 3 of *F. tricinctum* (0.2 to 6.0 μg). All isolates of *F. roseum* "Graminearum" which formed the perithecial stage of *G. zaeae* (*G. roseum*) produced zearalenone. Production occurred by the wild but not the aprassed cultural type. Zearalenone production by *F. tricinctum* was confirmed by a mouse bioassay.

The first report of moldy corn causing an estrogenic disturbance among swine appeared in 1928 (8). Since then, other reports have come from the United States (3, 5, 6, 19), Australia (16), and Ireland (7). The syndrome includes vulvar hypertrophy and occasional vaginal evison, preputial enlargement in castrated males, and prominent mammary glands in both sexes.

In 1962, Stob et al. (19) demonstrated that an anabolic uterotrophic compound, crystallized and partially characterized by them, was produced by *Gibberella zeae*, the perfect stage of *Fusarium graminearum* (*F. roseum* "Graminearum"). Urry et al. (20) determined the compound to be an enantomorph of 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone and named it zearalenone. Partial characterization of the compound and environmental conditions suitable for its production were reported by workers at the University of Minnesota (3, 10), who referred to zearalenone as F-2. Increased growth and shoot stimulation of tobacco pith callus tissue (11) are other reported effects of this compound.

Since many species of *Fusarium* attack portions of plants that are used as food or feed, the determination of species capable of zearalenone production is of importance. *F. culmorum*, *F. graminearum* (3), and *F. moniliforme* (12) were reported to be producers of this compound, whereas *F. lateritum*, *F. tricinctum*, *F. nivale*, *F. episphaeria*, *F. rigidiuscula*, *F. roseum*, and *F. solani* were not found to be producers (12). We report here an assessment of zearalenone production among *Fusarium* species and a sensitive chemical method for its determination.

**MATERIALS AND METHODS**

Sources of cultures. Identified cultures used in this investigation were received from investigators in the United States and from the American Type Culture Collection (Table 1). In addition, isolates were obtained from Indiana soil by plate dilutions on PCNB medium of Nash and Snyder (13) and from corn kernels plated on potato dextrose agar containing 100 μg of Tergitol NPX (a nonionic detergent, Union Carbide Corp., New York, N.Y.) per ml and 30 μg of chlortetracycline per ml. The latter two ingredients were added immediately before plate pouring.

Chemical screening procedure for zearalenone. Inoculum was grown in a shaken culture for 3 days at 24 C on carboxymethyl-cellulose medium (2). Inoculum (1 ml) was added to duplicate autoclaved samples (150 g each) of approximately 40% moisture corn and incubated for 3 weeks at 16 C. Selected isolates were also incubated at 16 C for 10 weeks or at 24 C for 2 weeks followed by 8 weeks at 12 C. Molded corn from each flask was extracted for 30 sec with 200 ml of anhydrous ethanol in a high-speed blender, an additional 200 ml of ethanol was added, and a second blending was performed. The liquid portion was decanted and filtered by suction through filter paper (Whatman no. 1). Anhydrous ethanol (200 ml) was added to the remaining solid particles, and the mixture was blended and filtered as above. The extracts

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1 Journal Paper no. 3890. Purdue University Agricultural Experiment Station.
2 Present address: Research Department, Commercial Solvents Corp., Terre Haute, Ind. 47808.
were refiltered and reduced to a syrup in a flash evaporator. The residue was extracted with two 50-ml portions of ethyl ether, which were combined and washed with 25 ml of deionized water. The aqueous phase was discarded. The ether solution was extracted three times with 25-ml portions of 0.25% NaOH. The alkaline extracts were combined, adjusted to pH 3.5 with 1 N HCl, and extracted three times with 25-ml portions of ethyl ether. The solvent was evaporated and the residue was dissolved in 1 ml of methanol. Silica gel G (Brinkman Instruments Inc.) thin-layer plates (250 μm) were prepared, dried for 2 hr at 103 C, and spotted with 5 μl of the methanol extract. The plates were developed in an unlined equilibrated tank by using a toluene-ethyl acetate-formic acid (TEF: 5:4:1) solvent system (17) and examined for zearalenone with shortwave ultraviolet light in a model C-5 Chromato-Vue chamber. Zearalenone has a Rf of 0.8 in this system. Semiquantitative estimates of zearalenone in the extracts were made by comparisons with graded amounts (0.5 to 2 μg) of authentic zearalenone (99.3 ± 0.6% pure as measured by phase solubility analysis, Tm = 163.2 C; lot no. 552552, Commercial Solvents Corp.). A 0.05-μg sample of authentic zearalenone can be detected on a thin-layer plate by this method.

Zearalenone recovery efficiency. Three of four 100-g samples of coarse-ground no. 2 yellow corn were fortified with 5 mg of authentic zearalenone. All samples were extracted by our previously described procedure and the extracts dissolved in 1 ml of methanol of spectrophotometric grade. Zearalenone extraction efficiency was judged as acceptable when 73% (average determination of 70, 74, and 75%) of the product was recovered, as measured by absorption at 236 nm. Spectrophotometric evaluation of extracts obtained by our method was ordinarily unsuitable because of interfering substances. The thin-layer procedure eliminated this difficulty and permitted assay without additional purification.

Perithecial production. As an aid in identification of species, all Fusarium isolates were checked for perithecial production on wheat culms. Two 4-cm sections of autoclaved wheat culms were aseptically placed on soil extract agar (9) slants, inoculated, and incubated under a 12-hr fluorescent light cycle at 23 C for 5 weeks.

Mouse bioassay for zearalenone estrogenicity. Three mouse bioassays were performed to: (i) check the validity of the TLC procedures used in the screening trial, (ii) test the ability of 2 F. moniliforme isolates to produce zearalenone, and (iii) to confirm the estrogenicity of F. tricinctum extracts. Extracts from corn cultures of two F. roseum "Graminearum" cultivars used in the screening trial were checked for estrogenicity. As in the screening trial, flasks of approximately 40% moisture corn were seeded with 1 ml of inoculum and incubated for 3 weeks at 16 C. Ethanol extracts of the corn were mixed with a ground corn-supplement ration and fed to groups of 10 ovariec-
tomized mice for 3 days. On the fourth day, the animals were killed and their uteri were weighed.

To insure a greater possibility of zearalenone production, two F. moniliforme isolates were incubated for 2 weeks at 24 C and then for 8 weeks at 12 C (10). Anhydrous ethanol extracts of 400 g of molded corn were added to 50 g of ground ration, air dried, and brought to 100 g with untreated feed. The

**Table 1. Production of zearalenone by Fusarium species on moist autoclaved corn kernels stored at 16 C for 3 weeks**

| Species          | No. of isolates producing zearalenone | No. of isolates tested |
|------------------|---------------------------------------|------------------------|
| F. episphearia   | 0                                     | 1                      |
| F. moniliforme   | 0                                     | 8                      |
| F. nivale        | 0                                     | 7                      |
| F. oxysporum     | 0                                     | 15                     |
| F. roseum        | 3                                     | 12                     |
| F. roseum "Acauminatum" | 0                         | 4                      |
| F. roseum "Avenaceum" | 3                                   | 6                      |
| F. roseum "Culmorum" | 3                                   | 3                      |
| F. roseum "Equiseti" | 3                                   | 6                      |
| F. roseum "Gibbosum" | 21                                   | 23                     |
| F. roseum "Graminearum" | 0                           | 1                      |
| F. roseum "Sambucinum" | 0                           | 9                      |
| F. solani        | 3                                     | 19                     |

a Name usually specified by the investigator from whom the culture was obtained. F. episphearia, collected by R. W. Caldwell from soil; F. moniliforme, collected by H. Leon-Gallegos, R. W. Caldwell, and P. B. Mislivec; F. nivale, collected by G. W. Bruehl and W. C. Snyder; F. oxysporum, collected by R. W. Caldwell, J. W. Lorbeer, D. R. Douglas, D. J. DeZeeuw, D. Davis, E. A. Curl, N. T. Powell, and H. W. Mussell (includes forma specialis batatas, cepae, lycopersici, melonis, nicotianae, and vasinfectum); F. roseum, collected by G. A. Bean, H. B. Couch, Gloria A. Rall, H. Cole, and R. L. Gabrielson (also includes the negative isolates of F. heterosporum ATCC 15625 and 15628); F. roseum "Acuminatum," collected by R. W. Caldwell and J. Tuie; F. roseum "Avenaceum," collected by R. J. Cook; F. roseum "Culmorum"; collected by W. C. Snyder and R. J. Cook (also includes ATCC 15620 collected by H. S. Pepin); F. roseum "Equiseti;" collected by R. W. Caldwell, D. M. Ma (includes ATCC 15622 collected by G. Kingsland); F. roseum "Gibbosum;" collected by R. J. Cook; F. roseum "Graminearum," collected by R. J. Cook, R. W. Caldwell, J. Tuie, W. C. Snyder, C. M. Christensen, L. E. Williams, Lois H. Tiffany, and A. J. Ullstrup; F. roseum "Sambucinum," ATCC 16552 collected by C. Booth; F. solani, collected by R. W. Caldwell and forma specialis phaseoli collected by C. R. Maier; F. tricinctum, collected by R. W. Caldwell, H. Cole, D. M. Ma, J. Tuie, J. J. Ellis, H. B. Couch, and E. B. Smalley. b Determined by thin-layer chromatography.
test rations were fed to 10 ovariectomized mice for 3 days.

The estrogenicity of three isolates of *F. tricinctum* was verified with corn cultures grown under the 10-week incubation regime described above. Anhydrous ethanol extracts of the molded corn were spotted on TLC plates. Methanol eluates from chromatoplates were dried and mixed with 5 ml of sesame oil, and 10 ovariectomized mice were injected subcutaneously with 0.1 ml of the mixture per day for 3 days.

**Extraction procedures for TLC used with *F. moniliforme***. Three isolates of *F. moniliforme* tested in the screening trial were grown for 2 weeks at 24°C and then for 8 weeks at 12°C. Both the procedure of Mirocha et al. and our procedure were used to detect zearalenone. The initial extracts for the procedure of Mirocha et al. (10) consisted of: (i) blending 100 g of molded corn with 400 ml of methylene chloride for 5 min and extracting for 16 hr or (ii) extracting 100 g of molded ground corn in a Soxhlet extractor with 400 ml of methylene chloride for 12 hr. The crude extracts were taken up in petroleum ether and partitioned with acetonitrile.

**RESULTS AND DISCUSSION**

**Screening of isolates for zearalenone.** Initially 110 isolates, belonging to 7 species of *Fusarium*, were screened for zearalenone production (Table 1). *F. tricinctum, F. roseum*, and the “Culmorum,” “Equisetii,” “Gibbosum,” and “Graminearum” cultivars of *F. roseum* produced zearalenone. The number of zearalenone-producing isolates and a broader range of production per gram of corn was as follows: 3 of *F. tricinctum* (0.2 to 6.0 μg), 3 of *F. roseum* “Culmorum” (1 to 210 μg), 3 of *F. roseum* “Equisetii” (0.6 to 2.0 μg), *F. roseum* “Gibbosum” (115 to 175 μg), and 21 of *F. roseum* “Graminearum” (0.2 to 230 μg). Zearalenone was produced by both of the duplicate samples tested, except in the case of one of the “Graminearum” and two of the “Equisetii” cultivars. Isolates which produced zearalenone were from different areas of the United States and Canada. Most of them were isolated from corn kernels, whereas others were from wheat, barley, turf grass, begonia, squash fruit, and soil. The estrogenicity of the extracts from two of the *F. roseum* “Graminearum” cultivars was verified with a mouse bioassay. A ethanol extracts of the two of the “Graminearum” cultivars induced uterine weights of 99.6 and 106.4 mg, as compared to 16.4 mg for the control.

Freshly isolated cultures of *F. roseum*, known as the wild type, generally grow rapidly with abundant aerial mycelium. Subculturing often leads to slower oppressed pionnotal growth and reduced plant pathogenicity (15). Of the 52 isolates of *F. roseum* screened for zearalenone, 8 grew oppressed. Since none of the oppressed cultures produced the toxin, it appears that zearalenone production is not associated with this cultural type.

Of the 21 wild-type isolates of *F. roseum* “Graminearum,” 19 produced the perithecial stage of *G. zeae*. None of the isolates of the other species produced perithelia. Since all 21 wild-type isolates of *F. roseum* “Graminearum” produced zearalenone, a close association between zearalenone production, perithecial formation, and the wild type in this taxon is indicated.

**Zearalenone production, estrogenicity, and morphology of *F. tricinctum***. Since *F. tricinctum* has not been reported to produce zearalenone, we attempted to verify its production by using an improved incubation protocol (10). Samples (150 g) of moist autoclaved corn were inoculated with *F. tricinctum* and incubated for 2 weeks at 24°C and then for eight weeks at 12°C. Extracts of eight replicates of each of the isolates suspected to produce zearalenone (isolates FT 2, 3, and 12) and two replicates of isolates that gave no evidence of toxin production (isolates FT 10 and 16) were prepared. All 24 extracts from isolates FT 2, 3, and 12 gave fluorescent spots with the same *Rf* value as zearalenone. By comparison with authentic standards, the average amounts of zearalenone were 9.4, 14.4, and 13.7 μg/g, respectively. These levels, although low, were considerably above the average levels of 3.5, 1.1, and 0.4 μg/g produced in the earlier trial. Extracts from isolates FT 10 and 16 showed no zearalenone.

To confirm the zearalenone production by isolates FT 2, 3, and 12, the spots on the chromatoplates corresponding to zearalenone were eluted with methanol (spectrophotometric grade) and the absorption spectra of the eluates were determined at 220 to 340 nm with a spectrophotometer (model DK-2; Beckman Instruments, Inc.). The absorption spectra closely resembled that of zearalenone with absorption peaks at 236, 274, and 314 nm.

The estrogenicity of the extracts from corn infected with three isolates of *F. tricinctum* was tested in a mouse bioassay. Extracts were injected into mice and gave significant increases in uterine weights with progressively greater average uterine weights with increasing zearalenone levels (Table 2).

The three zearalenone-producing cultures of *F. tricinctum* were isolated from soil, bluegrass, and corn kernels. The mycelium toward the center of the colony had a light-yellow tinge and the reverse was red with bands of pigmentation radiating from the center of the colony. Both macroconidia and microconidia were produced. The
TABLE 2. Effect of ethanolic extracts of Fusarium tricinctum on uterine weights of ovariecutomized mice

| Isolate no. | Zearalenone* | Uterine wt * |
|-------------|-------------|-------------|
| FT 2        | 3.1         | 78.0        |
| FT 3        | 2.7         | 58.3        |
| FT 12       | 2.1         | 50.3        |
| FT 10 + FT 16 | 0.0       | 23.2        |
| Standard    | 2.1         | 39.6        |
| Injected control | -      | 23.4        |
| Uninjected control | -      | 25.4        |

* Each isolate was grown in three portions of 150 g of corn (40% moisture), extracts were combined and concentrated, and zearalenone concentrations were determined (at 274 nm) from a standard curve.

* Average of 10 replications.

macroconidia had 1 to 3 septata and were unevenly tapered toward the tip. The microconidia varied from globose with a basal papilla to obclavate and were borne on bottle-shaped conidiophores. Morphologically and culturally, the zearalenone-positive and -negative isolates were indistinguishable.

**Further tests with F. moniliforme.** Mirocha, Christensen, and Nelson reported zearalenone production by *F. moniliforme*, but gave no yields (12). Because of the wide occurrence of *F. moniliforme* in corn kernels, further examination of this material was carried out. However, no zearalenone was detected by TLC by using the extraction and purification procedures of Mirocha et al., and us.

Two isolates of *F. moniliforme* were tested for estrogenic activity in a mouse bioassay. No significant increases in uterine weights were produced by extracts from *F. moniliforme*. The sensitivity of the bioassay was assumed to be at least 33 μg of zearalenone per g of feed since this level of authentic zearalenone, when administered for 3 days, gave a 44% increase in uterine weight. By inference, a response among the animals consuming the 100 g of feed treated with the extract from 400 g of molded corn would have resulted, if 8 μg of zearalenone/g was present in the original molded corn. Therefore, if zearalenone was produced by the two isolates of *F. moniliforme*, levels would have been below 8 μg/g.

Since zearalenone was produced by all the isolates of *F. roseum* "Graminearum" (*G. zeae*) that produced perithecia, we tested isolates of *G. fujikuroi*, the perfect stage of *F. moniliforme*. Isolates of *F. moniliforme* which form the sexual stage usually cause the banake disease on rice and produce gibberellins (18). Three isolates including ATCC 12616 and 12618 were grown on moist autoclaved corn at 24°C for 2 weeks and then for 8 weeks at 12°C. Extracts examined by TLC revealed no zearalenone.

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