Cultural Characteristics, Pathogenicity, and Zearalenone Production by Strains of *Gibberella zeae* Isolated from Corn

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ABSTRACT

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Isolates of *Gibberella zeae* from naturally infected corn kernels were classified into two groups based on colony morphology on potato-dextrose agar (PDA). The most frequently isolated strain (type A) grew rapidly and produced red-pigmented colonies with abundant aerial mycelium. In culture, this type produced varying amounts of zearalenone (1 to 433 mg/L) and was consistently pathogenic in field trials. In contrast, the type B strain was isolated less frequently (5% of isolations), grew slowly on PDA, and developed appressed brownish yellow colonies. Type B strains were nonpathogenic, but produced high levels of zearalenone in culture (up to 2,033 mg/L). Among pathogenic strains (type A), concentrations of zearalenone produced in infected ears were positively correlated with production in culture.

Additional key words: F-2 toxin, *Fusarium graminearum*.

Gibberella ear rot is an important disease of corn in the north central United States (7). The disease sometimes reaches epidemic proportions and can result in substantial economic loss not only in reduced grain quality, but also from toxicity to farm animals that eat contaminated corn (14,16). A common toxic response, hyperestrogenism, is induced by the metabolite zearalenone (6,10-hydroxy-6-*oxo-trans*-1-undecenyl)-β-resorcylic acid lactone), also known as F-2. Zearalenone is produced by *Gibberella zeae* (Schw.) Petch (= *Fusarium graminearum* Schw.) prior to and/or during storage (3,4,16), with contamination of cribbed corn being most common (2). *G. zeae* is commonly associated with members of the Gramineae and, in addition to stalk and ear rot of corn, it also incites crown rot and scab of barley and wheat and stem rot of carnation (1,10). Francis and Burgess (10) observed two naturally occurring and morphologically distinct forms of *F. graminearum* in Australia, which were associated, respectively, with either crown rots of wheat, barley, and oats, or aerial plant-part infections such as corn stalk rot, barley scab, and carnation stem rot.

Under optimal cultural conditions, extreme variation in zearalenone production has been reported among strains of *G. zeae* (2,8). In a study of corn kernels infected by *G. zeae*, which were collected during an ear rot epidemic in Ohio in 1975 and stored frozen, we isolated and evaluated the toxin production of 63 *G. zeae* strains (6). When plotted as cumulative probability of log-zearalenone levels, the magnitude of zearalenone production among isolates was log-normally distributed, with low producers predominating. Attempts to relate zearalenone production by particular isolates in culture to concentrations developing under field disease conditions have not been reported. However, in preliminary trials, we observed reduced pathogenicity among very high zearalenone-producing strains (>2,000 μg of zearalenone per gram of autoclaved corn), relative to moderate or low producers (6).

The present study describes two naturally occurring, morphologically distinct strains of *G. zeae* from corn and defines the relationship between virulence on inoculated corn and the production of zearalenone in culture and in corn ears in the field.

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MATERIALS AND METHODS

Most of the *G. zeae* strains used in this study were isolated directly from naturally infected field samples. Infected kernels were surface sterilized in 1% (v/v) aqueous sodium hypochlorite for 1 min, rinsed with sterile distilled water, and plated on potato-dextrose agar (PDA) containing 50 μg of chlorotetracycline per milliliter. Transfers were made from colony margins to PDA slants. Single conidial isolates prepared from the PDA slants were stored in heat-sterilized soil at 4 C.

For morphological comparisons and radial growth rate determinations, infested soil particles from stored single-spore cultures were spread on PDA, and after 4 days 5-mm-diameter agar plugs were transferred to the center of freshly prepared PDA (2% dextrose, w/v) plates. Colony morphology and diameter were recorded after 3 days of growth at room temperature (19–22 C). Five replicate plates were examined for each strain.

Perithecium formation was induced by using the carnation leaf disk technique (15).

Sixty-three isolates were obtained from stored (−5 C) corn samples and represented typical *G. zeae* strains present in infected ears from 19 Ohio counties (5). From 1977 to 1981, 135 additional strains were collected from similarly infected corn from Wisconsin and other north central United States locations. Reference strain 'Ascospore No. 55' was kindly supplied by C. J. Mirocha, Department of Plant Pathology, University of Minnesota, St. Paul.

In 1980 field trials at Arlington, WI, a single-cross corn hybrid (A634 × Mo17) susceptible to Gibberella ear rot was inoculated with 17 selected strains of *G. zeae*. The strains were chosen on the basis of morphological characteristics and zearalenone production in culture (Table 1). Ears were wound-inoculated with mycelium-infiltrated toothpicks 10 days following 90% silking (17). A randomized complete block design was used in which 20 different plants in each of four blocks were inoculated with each strain of the pathogen. Sterile toothpick inoculations served as controls. After 3 mo, ears were harvested, rated for percent rot, shelled, dried to 15% moisture content, and stored at −20 C prior to toxin analysis. Angular transformations of disease severity (percent rot) data were used for analysis of variance.

Because of the laborious and costly nature of zearalenone determinations, quantitative analyses were confined to one

randomly chosen experimental block. Shelled ears of each treatment in the block were combined, thoroughly mixed, and ground in a Wiley mill (4-mm-diameter screen openings). Duplicate 25-g samples were extracted with ethyl acetate and purified by liquid:liquid partitioning as described by Ikedobi et al (12). The purified extract was stored in 5 ml of chloroform. Recovery of zearalenone from spiked samples prepared in a similar manner was approximately 60%. All reported zearalenone concentrations are uncorrected for extraction losses.

Zearalenone produced in cultures by the various isolates was assayed by the Hidy method (11). Twenty-five grams of vermiculite-nutrient substrate were weighed into 10 × 100-mm petri plates, autoclaved for 15 min at 121 C, inoculated, incubated at 19 C for 3 wk, and stored at −20 C until analyzed. Without drying, vermiculite cultures were transferred to 500-ml Erlenmeyer flasks and extracted twice with 100 ml of ethyl acetate by agitating for 10 min on a rotary shaker (200 RPM). After filtration through Whatman No. 1 filter paper, the extracts were dried under vacuum, and the residue was redissolved in 100 ml of acetonitrile. The residue in acetonitrile was twice extracted with an equal volume of petroleum ether (BP 30–60 C) and dried under vacuum. The residue was taken up in 10 ml of acetone. The efficiency of zearalenone recovery from spiked cultures was 80%.

Before quantitation by gas-liquid chromatography (GLC), the trimethylsilyl (TMS) derivative of zearalenone was prepared by drying an aliquot of purified extract under nitrogen and adding 100 μl of silylation reagent (BT, Pierce Chemical Co., Rockford, IL 61105, or Derivisil, Regis Chemical Co., Morton Grove, IL 60053) in a Pierce Reactivial. After 10 min of incubation at 60 C, silylation was complete as determined by successive GLC injections.

In a model 1860 Varian gas chromatograph equipped with flame ionization detector (FID), 1-μl injections were made into glass columns (1.6 m × 2 mm) packed with 3% OV-17 on Chromosorb W (10/100-mesh) (Pierce Chemical Co.). A temperature program from 200 to 280 C (3 C/min) was used with the injector at 280 C and detector set at 300 C. Nitrogen and hydrogen flow rates were 30 and 20 ml/min, respectively. Quantitation was achieved by estimation of peak area relative to pure standards. Detector response to TMS-zearalenone was linear ($r^2 = 0.99$) between 0.05 and 2.0 μg.

Zearalenone levels in purified extracts of certain monosporic cultures (Table 2) were quantified on thin-layer chromatography (TLC) plates (0.25 mm thick) of silica gel G. Appropriate dilutions of purified extracts were prepared and 2-μl spots were developed with toluene:ethyl acetate:formic acid (5:4:1, v/v) in unlined tanks. Zearalenone concentration was estimated by fluorescence intensity relative to external standards under long (366 nm) and short (254 nm) wavelength ultraviolet light. Approximately 20–30% error (coefficient of variation) is associated with TLC determinations of zearalenone.

TABLE 1. Source and cultural characteristics of *Gibberella zeae* isolates used in 1980 corn ear rot field trials

Isolate designation	Cultural type[a]	Zearalenone[b] (mg/L)	Growth[c] (mm/3 days)
611-4	B	2,033	50.8 a
Crawford 5	B	602	50.2 a
Wood 1	B	193	55.8 b
Richland 2	B	65	48.6 a
Mahoning 1	A	146	71.8 de
Huron 3	A	132	81.6 h
Fairfield 2	A	33	70.6 d
Huron 2	A	28	66.4 c
Columbiana	A	11	72.6 def
Wyandote 1	A	10	75.4 efg
Hancock 2	A	7	73.4 defg
Crawford 1	A	5	73.4 defg
Wayne 1	A	2	65.0 c
Delaware 1	A	2	76.4 fg
Knox 1	A	2	72.3 def
Stark 2	A	2	77.2 g
Wyandote 2	A	1	74.4 defg

[a] Isolate 611-4 from Wisconsin, all others from Ohio. Type descriptions in text.
[b] Zearalenone production expressed as mean of four determinations.
[c] Mean 3-day colony diameter. Values followed by different letters are significantly different according to Tukey's multiple comparison test ($P = 0.05$).

RESULTS

Two distinct morphological forms of *G. zeae* were isolated from naturally infected corn kernels from the north central United States. The typical (type A) *G. zeae* isolates were fast growing (Table 1), produced abundant white aerial mycelium, and produced regular circular colonies on freshly prepared PDA (Fig. 1, top row). A red pigment that darkened with culture age, was apparent in the agar of type A isolates. In contrast, type B isolates were slower growing (Table 1), produced irregularly shaped appressed colonies on PDA, and were pigmented brownish yellow (Fig. 1, bottom row). Isolates of intermediate appearance and/or growth rate were not observed. All 17 isolates (type A and B) used in field inoculations (Table 1) produced fertile perithecia on carnation leaf disks.

Of approximately 200 isolations obtained for this study, 5% were type B. Three of these were isolated from Ohio and six from the north central United States. When multiple *G. zeae* isolations were made from several naturally infected ears, type B and type A isolates were found to occur together in one ear, whereas four ears yielded exclusively type A.

Type A isolates produced varying amounts of zearalenone whereas type B isolates were all high producers. For example, of the 17 isolates selected for the field trial (Table 1), type B isolates 611-4, Crawford 5, Wood 1, and Richland 1 produced 2,033, 602, 193, and 65 mg of zearalenone per liter of substrate, respectively. In addition, isolate CSC-542 (ATCC 20273), originally selected by Commercial Solvents Corporation (International Minerals Corp., Terre Haute, IN 47802) as an extremely high zearalenone producer (>1,000 mg/L) for use in commercial production, was a typical type B isolate (*unpublished*).

TABLE 2. Zearalenone production by single spore cultures of *G. zeae*

Parent isolate designation	Cultural[a] type	Spore type	Zearalenone production: Number analyzed	Mean (mg/L)	Standard error	Range
Crawford 5	B	Ascospores	50	720	224	334–1,042
Crawford 5	B	Conidia	50[b]	650	120	420– 850
Huron 3	A	Ascospores	50	357	43	272– 433
Huron 3	A	Conidia	50[b]	310	75	200– 420
M-1	A	Ascospores	15	31	6.8	24– 48
RC-3	A	Ascospores	15	26	7.5	18– 35
Ascospore No. 55[c]	A	Ascospores	36	36	9.9	25– 62
Ascospore No. 55[c]	A	Conidia	15	24	5.9	13– 32

[a] Morphological distinctions are described in the text.
[b] Thin-layer chromatography determinations; all others were by gas-liquid chromatography.
[c] Received as single ascospore derived cultures; all other cultures were mass mycelium isolates.

In the field trial of 17 isolates, significant ($P = 0.05$) differences in pathogenicity, unrelated to zearalenone production in culture, were observed among some type A isolates. Average disease severity for type A isolates ranged from 10% (Knox 1 inoculated) to 27% (Fairfield 2 inoculated) ear rot (Fig. 2). Mahoning 1, the highest zearaleneone producer among the type A isolates tested, produced approximately 15% ear rot. The four type B isolates tested were nonpathogenic (Fig. 2). No disease was observed on wounded control ears.

Among the 13 pathogenic isolates (type A) tested in the field, zearalenone content of infected ears was positively correlated ($r = 0.748$, $P = 0.01$) with production in culture (Fig. 3). In contrast, zearalenone content of infected ears correlated poorly with disease severity ratings ($r = 0.283$). Ears inoculated with type A isolates Mahoning 1 (point "b," Fig. 3) and Knox 1 (point "a," Fig. 3) contained highest (9 μg/g) and lowest (1.5 μg/g) zearalenone levels, respectively.

Single-ascospore and single-conidium cultures were analyzed for morphological stability and zearalenone production by using as parents either single-ascospore type A (Ascospore No. 55) or mass mycelial transfer type A (Huron 3, M-1, and RC-3) and type B (Crawford 5) isolates. All single-spore cultures were identical to parent strains with respect to cultural appearance and general level of zearalenone production (Table 2).

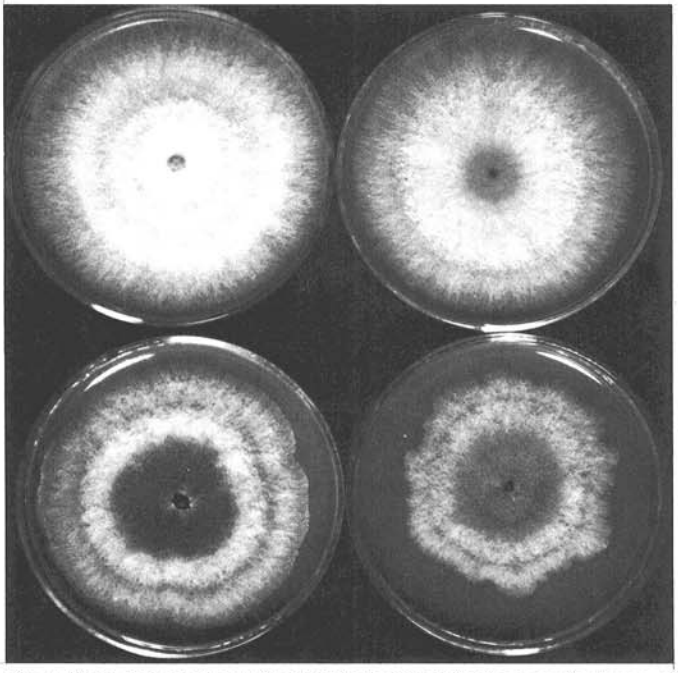

Fig. 1. Four-day-old type A (top row) and type B (bottom row) cultures of *Gibberella zeae* on potato-dextrose agar. All four isolates were from naturally infected corn and were used in 1980 field inoculation trials.

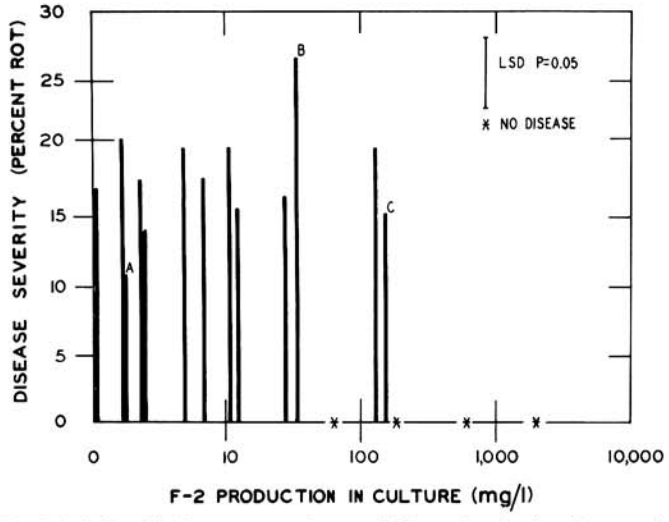

Fig. 2. Relationship between zearalenone (F-2) production in culture and pathogenicity of 17 isolates of *Gibberella zeae*. Bars and asterisks represent mean ear rot for individual strains listed in Table 1. Bars designated A, B, and C represent ears molded by isolates designated Knox 1, Fairfield 2, and Mahoning 1, respectively. Calculations of least significant difference (LSD) involved only the 13 pathogenic (type A) isolates ($P = 0.05$).

DISCUSSION

The genus *Fusarium* is well known for variability and the many problems encountered in maintaining its 'wild types.' The expectation of occasional cultural variation might explain why type B isolates have not previously been reported. Isolate distinctions described here bear some resemblance to group 1 and 2 *F. graminearum* strains reported by Francis and Burgess (10) on Australian wheat. For example, type A appears analogous to group 2 cultures in the abundance of aerial mycelium, pigmentation, and perithecia formation. Our type B isolates, however, produce abundant fertile perithecia in contrast to their group 1, which produce no perithecia. Type B isolates apparently bear little cultural resemblance to either group 1 or 2. The toxigenicity and pathogenicity of group 1 and 2 *F. graminearum* isolates described by Francis and Burgess (10) apparently have not been tested.

The origin and function of type B strains are problematic. Although less common than type A strains, type B frequencies of 5% seem far above the usual spontaneous mutation rates. Moreover, because of its slower growth relative to type A, an underestimation of type B prevalence is possible, especially when the two types occur together. Since conversions between type A and B were not observed in hundreds of single-spore isolations, segregations of hetrokaryotic nuclei or of extrachromosomal elements are unlikely explanations of their origin.

Under as yet undetermined conditions, type B strains may be pathogenic. The repeated isolation of type B strains from visibly diseased kernels from certain field samples supports this contention. Conceivably, environmental conditions not met during our field trials support some type B disease development. Alternatively, the two types might colonize together with type B actually dependent upon type A for growth. The isolation of both

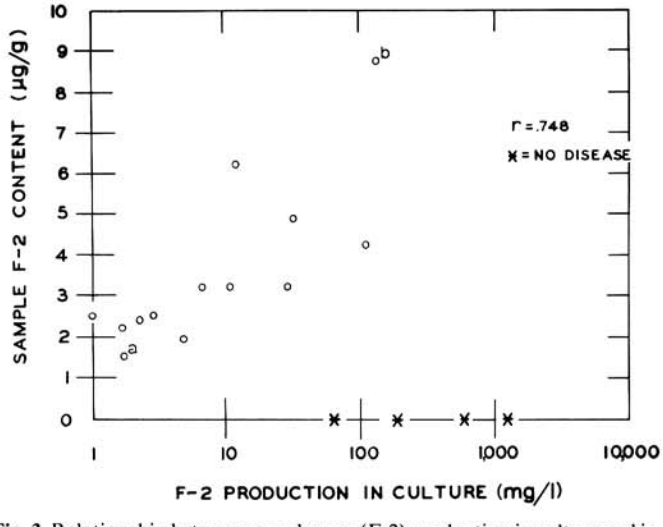

Fig. 3. Relationship between zearalenone (F-2) production in culture and in kernels from ears inoculated with *Gibberella zeae*. Each point represents individual type A (O) or type B (*) isolates listed in Table 1. Points designated a and b represent samples molded by strains Knox 1 and Mahoning 1, respectively. F-2 levels in infected ears (sample F-2 content) averaged for duplicate samples. Calculation of correlation coefficient (r) involved only the 13 pathogenic isolates (type A). The correlation ($r = 0.748$) was highly significant ($P = 0.01$).

types from a single infected ear in these studies suggests this mode of survival of type B in nature. To help resolve this issue, experiments involving simultaneous inoculations with both types are being contemplated.

Although periods of cool wet weather following silking favor *G. zeae* ear infection (16), the etiology of zearalenone toxicosis is still largely unknown. Episodes of zearalenone-induced hyperestrogenism do not necessarily follow reports of heavy field infection (13,14). Moreover, visual estimates of *G. zeae* invasion of suspect feed samples are inaccurate predictors of toxin content. Presumably, environmental conditions other than those favoring fungal invasion are required for toxin production. Mirocha et al (9,13) have demonstrated low-temperature (12–15 C) enhancement of zearalenone synthesis.

We suggest that variations in strain pathogenicity and toxin production play an important role in zearalenone toxicosis. The positive correlation between field production and cultural production by pathogenic strains supports this thesis. If sufficiently colonized by a high-producing strain, a single ear could contain several milligrams of zearalenone at harvest. This concentration would be expected to increase during storage, especially under cool, wet conditions. Consequently, ingestion of that single ear could readily induce an estrogenic response, particularly in swine. Individual strain effects would be less severe in bulk-shelled corn in which dilution of toxic kernels would occur.

Before selecting corn lines resistant to zearalenone synthesis, the pathogenic and toxigenic variability of strains of *G. zeae* should be recognized, and combinations of mixed inoculum sources, including both A and B type strains, should be used in screening programs.

Finally, the association of high zearalenone production with type B cultural morphology should be useful in the selection of high-producing strains.

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