Distribution of Trichothecenes and Zearalenone in *Fusarium graminearum*: Rotted Corn Ears Grown in a Controlled Environment

Glenn A. Bennett,* Donald T. Wicklow, Rodney W. Caldwell, and Eugene B. Smalley

Pathogenic and toxigenic properties of nine strains of *Fusarium graminearum*, isolated from maize (*Zea mays* L.) expressing symptoms of ear rot, were evaluated. Ears of a commercial dent corn hybrid (DeKalb XL-12) were wound-inoculated 7 days after silking and grown to maturity in a controlled-environment facility (Biotron, University of Wisconsin). Mycotoxin levels were determined in kernels from three visibly distinct zones of infected ears. The highest toxin levels always occurred in the severely rotted zone. Zone I kernels contained 62.0–162.3 μg/g deoxynivalenol (DON), 6.1–17.0 μg/g 15-acetyldeoxynivalenol (15-AcDON), and 2.5–4.8 μg/g zearalenone. Zone II kernels contained 1.1–4.6 μg/g DON, 0.2–0.8 μg/g 15-AcDON, and 0.1–1.9 μg/g zearalenone. Undamaged kernels (zone III), which exhibited symptomless infection (12–66% infected kernels), contained less than detectable amounts (0.05 μg/g) of trichothecenes and zearalenone. Severely rotted cobs contained 42.3–149.6 μg/g DON, 6.7–23.1 μg/g 15-AcDON, and traces of zearalenone. Visibly undamaged sections contained 0.1–2.8 μg/g DON and 0.10–0.3 μg/g 15-AcDON; zearalenone was not detected. Removal of the heavily damaged kernels would significantly reduce the risk of animal mycotoxicoses when such corn is used as feed.

Fusarium is a genus of ubiquitous fungi, and several species are important pathogens of cereal crops (Booth, 1971). In addition to causing severe crop yield reduction, some of these species may produce mycotoxins of concern to both livestock and human health (Ueno, 1983; Trenholm et al., 1983). One such fungus, *Fusarium graminearum* Schw. [*Gibbrella zeae* (Schw.) Petch.], causes ear rot of preharvest corn and also contaminates the kernels with mycotoxins (Miller et al., 1983a; Atlin et al., 1983). Typically, ear rot is initiated through natural wounds (i.e., insects or birds) at the tip of the ear and moves down the ear as a contiguous mold front (Sutton et al., 1980). This pattern of rotting results in zones of differing kernel rot severity along the ear. The present study reports on the types and levels of *F. graminearum* mycotoxins in kernels from three visibly identifiable zones of rotted ears as well as underlying portions of the cobs. Kernel and cob samples were analyzed for deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-AcDON), nivalenol, fusarenon-X, diacetoxyscirpenol (DAS), T-2 toxin, and zearalenone. It is important to understand how each zone contributes to the overall mycotoxin profile of individual rotted ears in order to guide technological approaches for removing or segregating significant portions of the toxin-contaminated grain.

MATERIALS AND METHODS

Experimental Design. A commercial dent corn hybrid (DeKalb XL-12) was grown to maturity in the University of Wisconsin Biotron under environmental conditions described by Caldwell et al. (1984). Temperature and photoperiods were 30 °C for 14 h (days) and 20 °C for 10 h (nights). Relative humidity was maintained at 82 ± 5%. Ears were inoculated with nine single-spored isolates of *F. graminearum* that produced zearalenone and trichothecenes (DON, 15-AcDON) in culture. Seven days after silking, three infected toothpicks were inserted equidistant near the top of each ear. Ears were harvested at 104 days after planting with kernel moisture at 9.5–15%. Three zones of visibly distinct kernel rot were identifiable: I, severe rot (kernels dull brownish red with no bright yellow aleurone layer present); II, light to moderate rot (kernels with at least a portion of the bright aleurone layer present); III, no rot (all kernels sound and bright yellow).

Kernels from each of the three zones (three ears per sample) were separately shelled from the ears and assayed for mycotoxin content. Two zones of damage were discernible in the underlying cob: I, rotted; III, sound cob. All samples were stored 0 °C until analyzed. Representative kernels (50) from zone III (sound) were surface-sterilized for 2 min in 2% Cholorox plus 0.01% Triton X-100 and plated onto potato–dextrose agar. The percent infection by *F. graminearum* was determined after 7 days.

Source and Characterization of Isolates. Single-spored isolates of *F. graminearum*, maintained on desiccated silica gel, were tested for types and quantities of mycotoxins produced in laboratory cultures. Each isolate was cultured on autoclaved, cracked corn (toxin free) for 21 days at 25 °C. These cultures were assayed for DON, 15-AcDON, nivalenol, fusarenon-X, DAS, T-2 toxin, and zearalenone as outlined below. With the exception of E-4, all strains were isolated from rotted ears collected in the fall of 1975 in northern Ohio. The isolates were designated by their county of origin. Culture E-4 was isolated from corn collected in Manitowoc County, WI, in 1979. All are deposited in the Agriculture Research Service Culture Collection (NRRL), Peoria, IL, as lyophilized cultures.

Mycotoxin Analyses. Corn kernels from the three zones (I–III) were accurately weighed prior to analyses. Typically, 25-g samples were used for assay. Because zone I kernels did not amount to this quantity, the entire sample (12.5–22 g) was used for analysis. Mycotoxins were extracted by blending samples in a Waring blender for 5 min with 200 mL of chloroform–methanol (1:1). The suspension was centrifuged for 10 min at 7000 rpm, and the supernate was filtered through rapid-flow filter paper. The precipitate was reextracted with a second 200-mL volume of solvent and centrifuged, and the filtrates were combined. A volume of extract containing a 10-g equivalent of initial sample was dried under reduced pressure and dissolved in methanol (1–2 mL), and then acetonitrile (25 mL) was added. This solution was defatted with two 50-mL volumes of hexane, and the acetonitrile was then removed under reduced pressure. The residue was dissolved in 2 mL of chloroform–methanol (9:1) and transferred to a silica gel (1% water) column prepared by layering, in sequence, anhydrous sodium sulfate (1 g), silica gel (5 g), and an-

Northern Regional Research Center, U.S. Department of Agriculture—Agricultural Research Service, Peoria, Illinois 61604 (G.A.B., D.T.W.), and Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706 (R.W.C., E.B.S.).

Table I. Production of Deoxynivalenol, 15-Acetyldeoxynivalenol, and Zearalenone on Corn by *Fusarium graminearum* in Laboratory Cultures

strain designations		mycotoxin,[a] μg/g		
NRRL[b]	county[c]	DON	15-AcDON	zearalenone
13193	Stark-B	35	8	259
13173	Fairfield-B	360	32	119
13172	E-4	52	24	150
13176	Fulton-B	35	5	29
13181	Huron-C	73	11	800[d]
13184	Mahoning-A	75	10	802
13180	Huron-B	43	10	280
13188	Richland-B	87	15	41
13183	Liking-B	195	35	713

[a] Average of results from duplicate samples. [b] NRRL = Northern Regional Research Laboratory, Agriculture Research Service Culture Collection Designation. [c] County of origin designation. [d] Huron-C also product α-zearalenol (1.20 μg/g).

hydrous sodium sulfate (2 g). The column was washed with toluene (100 mL), and zearalenone was eluted with 100 mL of toluene–acetone (95:5); 15-AcDON with 100 mL of chloroform–methanol (98:2); and DON with 150 mL of chloroform–methanol (95:5). Each fraction was dried under reduced pressure, transferred to 4-mL screw-capped vials with chloroform–methanol (9:1), and then dried under nitrogen.

Damaged and undamaged cob sections were course ground in a Wiley mill, and 25-g samples were extracted by shaking for 2 h on a wrist-action shaker with 200 mL of chloroform–methanol (1:1). The extracts were filtered, the solvent was removed under reduced pressure, and the residues were cleaned up by column chromatography as described for kernel samples.

Quantitation of Mycotoxins. Dried residues containing trichothecenes were dissolved in 4 mL of toluene–acetonitrile (95:5), and the heptafluorobutyrate derivatives were prepared as described by Bennett et al. (1983). Analyses were carried out on a Spectra-Physics Model 7100 gas chromatograph with an electron capture detector. Chromatographic parameters: column, 4 ft × 2 mm, packed with 3% OV-1 on Gas Chrom Q 100/120 mesh; oven temperature, 150 °C for 5 min and then programmed to 250 °C at 10°/min; carrier gas, nitrogen at 60 mL/min; injector 210 °C; detector, 350 °C.

Zearalenone and α-zearalenol were determined by HPLC of the toluene–acetone (95:5) fraction from the silica column as described by Bennett et al. (1985). All column fractions were tested to determine presence of other toxins and to determine the efficiency of separations. The identity of 15-AcDON was established by TLC and mass spectrometry.

Quantitation of Ergosterol. Samples of corn kernels from zone III (undamaged) were assayed for ergosterol by following the HPLC procedure outlined by Miller et al. (1983a).

RESULTS AND DISCUSSION

Toxin Production in Laboratory Cultures. Table I shows the levels of DON, 15-AcDON, and zearalenone produced on cracked corn by isolates subsequently used in the Biotron experiments. DON levels ranged from 35 to 360 μg/g (mean 106 μg/g). Levels of 15-AcDON, a presumed precursor of DON (Pestka et al., 1985; Miller et al., 1983a), ranged from 5 to 35 μg/g (mean 17 μg/g). No obvious correlation is apparent between levels of DON and 15-AcDON produced by these strains when grown on cracked corn for 21 days. Zearalenone levels appear unrelated to trichothecene levels and were higher than levels of DON in six of the strains tested. Only one strain (Huron-C) produced α-zearalenol (1.2 μg/g). Other toxins reported to be produced by *F. graminearum* but not detected in extracts of these cultures included nivalenol, fusarenon-X, DAS, and T-2 toxin. Miller et al. (1983b) reported that six of twelve *F. graminearum* isolates of Canadian origin produced DAS and T-2 in trace amounts; however, those isolates were grown in liquid media.

Toxin Levels in Corn Kernels and Cobs. This is the first report of the development of *F. graminearum* (Gibberella) ear rot in a controlled environment (Biotron). In this artificial environment, as in nature, the fungus progressed down the ear and resulted in visibly distinct zones of ear rot damage. Zone I (heavily rotted kernels) contained the highest levels of DON (62.0–162.3 μg/g) (Table II). In this zone, all nine isolates of *F. graminearum* also produced 15-AcDON within a narrow concentration range (6.1–17.0 μg/g). Levels of toxins dropped dramatically in zone II (light to moderate rot) kernels. Contrasted with zone I, DON levels were 6–117 times less (1.1–4.6 μg/g) and 15-AcDON levels were 8–94 times less (0.2–0.8 μg/g). No toxin could be detected in the sound kernels from the rotted ears; however, mycological examination indicated *Fusarium* infection of 12–66% of the kernels sampled.

The levels of DON and 15-AcDON in *F. graminearum* rotted cobs (zone I) and undamaged cobs (zone III) are listed in Table III. The cobs were divided into only two categories (damaged, sound) due to the difficulty in distinguishing where the cob rot damage terminated. The white pith below sound cob was occasionally discolored when compared to healthy cob sections. Careful segregation was, therefore, not feasible, and the lower (base end) one-third of the cob was separated and called zone III, which corresponds to kernel zone III. Successive extractions with 200-mL portions of chloroform–methanol (1:1) indicated that a minimum of 2 h was required to extract cobs by shaking. Cobs, even when broken in small pieces, do not lend themselves to extraction by blending due to their buoyancy. The ratio of 15-AcDON to DON was slightly higher in the cob than in the corresponding kernels. The levels found in the "sound" cob could have resulted from incomplete segregation of rotted from healthy cob and possibly from translocation of toxins that can occur in infected corn plants (Sutton et al., 1976).

The levels of zearalenone found in the different zones of *F. graminearum* rotted ears were low when compared with levels we detected in laboratory cultures of those strains on cracked corn (Tables I and IV). However, these levels are similar to levels reported for *F. graminearum* inoculated field corn at maturity (Miller et al., 1983a; Atlin et al., 1983). In the heavily damaged kernels, only one sample exceeded 4.0 μg/g (Table IV). Very consistent levels, 2.6–3.6 μg/g, were found in the other eight samples from inoculated ears. No zearalenone could be detected in sound (zone III) kernels or cobs. These data also corroborate Caldwell and Tuite's findings (1970, 1974) that low (<5 μg/g) levels of zearalenone are present in corn at harvest. Ergosterol was detected in sound kernels (zone III), but at relatively low levels (0.2–2.0 μg/g), which indicates low fungal contamination of these kernels. We did not determine ergosterol levels in zone I or II.

Comparison of mycotoxin profiles from laboratory cultures with those from samples of rotted kernels from Biotron-grown ears at harvest shows no difference in mean levels of DON (culture, 106 μg/g; Biotron, 115 μg/g). At the same time, substantial differences in mean levels of zearalenone (culture, 354 μg/g; Biotron, 3.0 μg/g) were recorded for these samples. Temperature differences were probably not responsible for these results. The incubation

Table II. Distributions and Levels (μg/g) of Deoxynivalenol and 15-Acetyldeoxynivalenol in Kernels from Ears Inoculated with *F. graminearum* Isolates

NRRL strain designations	ear zones[a] I DON	I AcDON	II DON	II AcDON	III DON	III AcDON	% infected kernels (III)
13193	99.4	15.5	4.6	0.7	ND	ND	66
13173	147.2	12.3	3.4	0.3	ND	ND	22
13172	103.0	17.0	2.5	0.7	ND	ND	42
13176	127.7	15.1	1.1	0.2	ND	ND	32
13181	105.8	13.6	2.2	0.3	ND	ND	12
13184	62.0	6.1	1.9	0.8	ND	ND	44
13180	93.1	7.5	1.4	0.2	ND	ND	36
13188	162.3	8.1	2.8	0.3	ND	ND	64
13183	136.8	8.2	2.4	0.2	ND	ND	30

[a] Zones: I, heavily rotted kernels; II, lightly to moderately rotted kernels; III, sound kernels.

Table III. Distribution and Levels (μg/g) of Deoxynivalenol and 15-Acetyldeoxynivalenol in Cobs from Ears Inoculated with *F. graminearum* Isolates

NRRL strain designation	cob zone[a] I DON	I AcDON	III DON	III AcDON
13193	138.6	23.1	0.6	0.1
13173	90.1	17.8	0.5	0.1
13172	42.3	13.2	1.0	0.3
13176	149.6	22.1	0.4	0.1
13181	74.1	11.1	0.2	0.1
13184	102.4	14.7	0.1	0.1
13180	48.4	7.4	2.8	0.3
13188	63.1	6.7	0.5	0.3
13183	52.0	9.4	1.0	0.3

[a] Zones: I, cob section underlying heavily damaged and moderately damaged kernels; III, cob section underlying undamaged kernels.

Table IV. Distribution and Levels (μg/g) of Zearalenone in Kernels and Cobs from Ears Inoculated with *F. graminearum* Isolates

NRRL strain designation	kernel zones[a] I	II	III	cob zones I	III
13193	2.8	1.0	ND	0.02	ND
13173	2.9	1.3	ND	0.02	ND
13172	2.8	0.1	ND	ND	ND
13176	2.5	1.0	ND	0.02	ND
13181	4.8	1.9	ND	0.03	ND
13184	2.8	0.1	ND	ND	ND
13180	3.6	1.5	ND	0.03	ND
13188	2.6	1.0	ND	ND	ND
13183	2.6	0.7	ND	ND	ND

[a] See Table II for zone designations.

temperature for laboratory fermentations was 25 °C, while daily Biotron temperatures were programmed for 30 °C day/20 °C night.

CONCLUSIONS

These studies demonstrate that trichothecene and zearalenone production occurs almost exclusively in heavily damaged and moderately damaged corn kernels and underlying cobs. Sharp gradients exist in toxin levels in the three visually distinct zones or areas of *Fusarium* damage. Due to the "utilization" of the corn matrix by the invading pathogen, toxin-containing kernels are significantly lighter than uncontaminated kernels. Significant removal of damaged kernels by density segregation has been demonstrated by Huff and Hagler (1985). They reported that removal of deoxynivalenol-contaminated corn kernels, buoyant in water and 30% sucrose, reduced toxin levels by 53 and 77%. During harvest, a picker–sheller could remove a good portion of the heavily damaged kernels and would thus remove a significant portion of the toxins. Removal of these kernels at harvest would significantly reduce the risk of animal mycotoxicoses when such corn is used as feed.

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Registry No. DON, 51481-10-8; 15-AcDON, 88337-96-6; zearalenone, 17924-92-4.

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Metabolism of Methabenzthiazuron in the Soil of Pea Crops

Jean Rouchaud,* Pascal Roucourt, Michel Van Himme, Frans Benoit, Norbert Ceustermans, Joël Gillet, Willy Plumier, and Georges Vulsteke

The herbicide methabenzthiazuron (MBT) was presowing applied onto the soil of pea fields in four regions with three different soil types. MBT, (2-benzothiazolyl)urea (IV), 2-(methylamino)benzothiazole (V), and 2-aminobenzothiazole (VI) were observed in all soil types. Concentrations of compound IV in soil greatly varied with the soil type. The amines V and VI represented a majority of the soil bound residue. The half-life in soil of MBT was 2 months when concentration of compound IV was low and 1 month when it was high. The half-life of the total of ureas was 2 months in all regions, and that for the total of ureas + identified metabolites was 6 months.

The urea derivative methabenzthiazuron [1,3-dimethyl-3-(2-benzothiazolyl)urea, MBT, I] is a very effective herbicide in grain and certain vegetable crops including peas. However, MBT has a rather long life in soil and may, by its remnant residues, be phytotoxic to some crops grown after the one for which the herbicide was applied (Van Himme et al., 1984). Several studies have been reported on the fate of MBT in soil, under laboratory conditions. After a [^{14}C]MBT-treated soil was aged for 6 months, the amount of ^{14}C activity remaining in the soil was equivalent to 50–60% of the MBT originally applied (Cheng and Führ, 1976). Only 50–70% of the ^{14}C remaining in the soil could be extracted; however, over 90% of the ^{14}C in the extracts was identified as the parent MBT. The main metabolite in the extract (5% of its ^{14}C content) was 1-methyl-1-(2-benzothiazolyl)urea, II (Mittelstaedt et al., 1977). However, low concentrations of 1-methyl-3-(2-benzothiazolyl)urea (III) may also be present in the soil extract, as III was formed during in vitro microbial decomposition of MBT (Wallnöfer et al., 1976; Goettfert et al., 1978).

In the present study, the fate of MBT in the soil of pea crops grown in four different regions of culture is studied. Identification and measurement of extractable and bound residues of MBT for up to 6 months after treatment with herbicide MBT are reported here.

EXPERIMENTAL SECTION

Pea Crops and Treatments. The peas (cv. Minarette, used for canning) were grown in fields located in three regions of culture different as to their soil types (Table I). The soil characteristics were determined in 1986. Each field was subdivided into four plots corresponding to four replications. MBT was applied to soil just before sowing the peas at a rate of 2 kg of MBT/ha by spraying an emulsion of Tribunil (wettable powder obtained from Bayer Belgium, containing 70% w/w MBT) in water (400 L/ha). MBT was applied on top of soil and incorporated into the top 3 in. of soil, and then the seeds were sown. At harvest, samples of about 1 kg of dehulled peas were collected at random from each plot.

Thin-Layer (TLC) and Gas–Liquid (GLC) Chromatographies, Mass (MS) and Infrared (IR) Spectrometries, and Nuclear Magnetic Resonance (NMR). TLC was performed using DC-Plastikfolien Kieselgel 60F254 plates, 20 × 20 cm and 0.2 mm thick, Merck. All the mobile-phase solvents described by Cheng and Führ (1976) were used, some of them successively, to resolve MBT and its metabolites. The R_f with the eluting solvents generally used were as follows. (1) Chloroform: I, 0.25; II, 0.17; III, 0.05; IV, 0.03; V, 0.16; VI, 0.10. (2) Ethyl acetate: I, 0.64; II, 0.63; III, 0.45; IV, 0.45; V, 0.68; VI, 0.64. Acetonitrile: I, 0.87; II, 0.88; III, 0.81; IV, 0.78; V, 0.82; VI, 0.73. Identification was done by single-dimensional TLC development. With the mobile-phase solvents giving low R_f values, after first development the TLC plate was dried and redeveloped as such. This procedure was repeated several times, making it possible to resolve the standards when applied as a mixture onto the TLC plate.

GLC was performed with a Tracor 550 apparatus, using a flame photometric detection. Injection port and detector temperatures were at 300 and 180 °C, respectively. Glass column (1.80 m × 2 mm (i.d.)) filled with 1% OV17 + 5% OV210 on 80–100-mesh Gas Chrom Q was used. Nitrogen carrier gas was used at a flow rate of 30 mL/min. Retention times for 2-aminobenzothiazole (VI) and for 2-(methylamino)benzothiazole (V) were 2.7 and 3.4 min, respectively. By the GLC, MBT and II were detected as V; III and (2-benzothiazolyl)urea (IV) were detected as VI.

Mass spectra were reached with the Varian MAT 311 (70 eV; m/e, relative abundance, %) apparatus employed in the electron impact (spectra described here) and chem-

Laboratoire de Phytopathologie, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium (J.R., P.R.), Research Center for Weed Science, Rijksuniversiteit Gent, 9000 Gent, Belgium (M.V.H.), Research Center for Vegetables, 2580 St Katelijne-Waver, Belgium (F.B., N.C.), School for Horticulture, 5800 Gembloux, Belgium (J.G.), Research Station for Vegetables, 5800 Gembloux, Belgium (W.P.), and Departemental Research Station for Horticulture, 8810 Roeselare-Rumbeke, Belgium (G.V.).

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