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Natural Occurrence of the Mycotoxin Fusarochromanone, a Metabolite of *Fusarium equiseti*, in Cereal Feed Associated with Tibial Dyschondroplasia

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The mycotoxin fusarochromanone, a metabolite of *Fusarium* fungi, is able to induce tibial dyschondroplasia (TD) in chickens under experimental conditions. On the basis of health surveillance data on TD, two broiler farms with TD prevalence rates of up to 56% were identified. In the corresponding pelleted feed samples, fusarochromanone was detected in all 12 samples analyzed by column purification and TLC, with concentrations 4 to 59 μg/kg. No *Fusarium* fungi were available from the feed because of the pelleting process, but seven *Fusarium equiseti* strains previously isolated from Danish cereals were checked for fusarochromanone production, and all produced fusarochromanone at 57 to 1,435 mg/kg. Thus, the potential for fusarochromanone production by *F. equiseti* is considerable. The identification of fusarochromanone from feed and *F. equiseti* was confirmed by mass, infrared, and nuclear magnetic resonance spectral analyses. This is the first report of fusarochromanone as a naturally occurring contaminant.

Avian osteochondrosis is a defect in endochondral ossification which affects primarily the tibiotarsal bones of chickens and turkeys. Morphologically, it is characterized by a cone of cartilage which extends distally from the proximal physis and is not penetrated by metaphyseal vascular sprouts (4, 8–10, 14). The defect is not limited to the tibiotarsus but also involves the tarsometatarsus, humerus, and femur (9, 10). However, as the changes generally are most pronounced in the tibiotarsus, the term tibial dyschondroplasia (TD) has been coined (6), and this term will be used throughout this report.

TD occurs commonly all over the world (1, 18). Several factors have been considered to play a role in the pathogenesis of the disease, such as genetics (11), the calcium-phosphorus ratio (2, 12), the chloride-sodium-calcium ratio (13), and management (15). More recently, a mycotoxin has been suggested as the cause of the disease. Thus, TD was induced in broiler chickens fed a diet containing as little as 2% of a *Fusarium roseum* culture (16, 17). Further investigations have indicated that the fungal organism is *F. equiseti* (19). Subsequently, a water-soluble component called TDP-1 was isolated from a *Fusarium* culture and purified, and this compound was able to induce TD in all of the chickens tested during a 3-week feeding period (5). Structural studies of the toxic compound using nuclear magnetic resonance (NMR) spectral, infrared (IR) spectral, and mass spectrometric (MS) data have indicated that the mycotoxin is a chromanone derivative named fusarochromanone (7). In this report, we describe the natural occurrence of fusarochromanone in cereal feed, the association with field cases of TD, and the fungal potential for fusarochromanone production of *F. equiseti* strains isolated from Danish cereals.

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MATERIALS AND METHODS

Pathologic changes in poultry. Prevalence data for TD were obtained from two broiler farms covered by a health surveillance program. A random sample of 16 live birds and 3 to 16 dead birds was collected at different times during the rearing period. At slaughter, a random sample of condemned chickens was obtained at the slaughterhouse. The chickens were examined for macroscopic TD changes by a sagittal cut through the proximal epiphysial growth plate of the tibia.

Poultry feed. Samples of feed (200 g) were collected six times during the rearing period, corresponding to the number of times feed batches were delivered to the farms. The feed was a pelleted product composed of a number of components, the major ones being wheat (44%) and corn (15%) or peas (5 to 15%). Attempts to isolate the original mycoflora of the feed samples, including *Fusarium* species, were unsuccessful, most likely because the original mycoflora had been heat destroyed during the pelleting process.

Fungi and fungal production of fusarochromanone. Seven strains of *F. equiseti* (Corda) Sacc. had been isolated previously from cereals (six strains) and peas (one strain). The strains were identified with the nomenclature of Gerlach and Nirenberg (3). One-liter Erlenmeyer flasks containing 200 g of polished rice and 100 ml of water were autoclaved at 121°C for 45 min. The flasks (two per strain) were inoculated with spores of the seven *F. equiseti* strains from Danish sources, as well as spores of one known fusarochromanone producer, *F. equiseti* Alaska 2-2 (supplied by Chester J. Mirocha, University of Minnesota, St. Paul). The flasks were incubated as stationary cultures at room temperature (18 to 20°C) for 1 month. Subsequently, the cultures were harvested, transferred to paper bags, dried at room temperature for 2 days, and maintained at −20°C until use.

Chemical analysis for fusarochromanone. Poultry feed and rice culture samples were analyzed by a previously published, slightly modified procedure (5, 7). Thus, 200 g was

extracted five times with chloroform-methanol-ammonium hydroxide (90:10:1) and filtered. The filtrates were pooled, concentrated in vacuo to dryness, and dissolved in 100 ml of methanol-water (1:4). This mixture was further concentrated to 60 ml and applied directly to a column packed with Amberlite XAD-2 resin. The column was rinsed with 450 ml of distilled water and eluted with 400 ml of 90% aqueous methanol. The methanol eluate was concentrated in vacuo, dissolved in 20 ml of chloroform-methanol (9:1), and mixed with 20 g of anhydrous sodium sulfate. After the powder was dried at room temperature, it was packed on the top of a column containing 60 g of Florisil. Successive elution was carried out with 800 ml of chloroform-methanol (9:1), followed by 800 ml of chloroform-methanol-ammonium hydroxide (90:10:1). Fusarochromanone was detected by irradiating the column with longwave (365-nm) UV light, since the toxin produces intense blue fluorescence under UV light. The fractions containing fusarochromanone were concentrated (40°C) and subsequently purified by thin-layer chromatography (TLC) with chloroform-methanol-ammonium hydroxide (80:20:2). Identification and quantitation were carried out with this mobile phase by TLC using reference fusarochromanone. Viewed in the dark under longwave (365-nm) UV light, fusarochromanone appears as a blue fluorescent spot at $R_f = 0.5$. Quantitation was performed by comparing the fluorescent intensities of fusarochromanone spots in samples with those of reference spots, determining sample and reference materials which matched closely, and interpolating if necessary. The least amount of fusarochromanone that can be detected as a spot on TLC plates is 0.6 ng. Recovery of 72.8% was obtained by analysis of three samples of barley spiked with 100 μg of fusarochromanone per kg.

Confirmation of fusarochromanone identity. Purified material, identified as fusarochromanone by TLC, from a rice culture of *F. equiseti* 1523 (sample I) and combined fusarochromanone material from farm B feed samples (sample II) were subjected to confirmatory analysis. MS analysis was conducted on both samples I and II, whereas IR and NMR spectral analyses were conducted only on sample I, as the amount of fusarochromanone in sample II was too small to provide useful data.

MS data were obtained by a VG Masslab 20-250 instrument equipped with an HP 5890 gas chromatograph. The fused silica WCOT OV-101 column (16 m by 0.20 mm; 0.6-μm pore size) was programmed from 40 to 250°C at 15°C/min to 100°C and kept there for 4 min, followed by a rate of 30°C/min to 250°C. The end of the gas chromatography column was inserted through a heated transfer line into the ion source. This line was kept at 300°C. The injector temperature was 250°C. The mass spectrometer was operated in the electron ionization mode with an electron energy of 70 eV, an emission current of 200 μA, and an ion source temperature of 200°C.

The NMR spectra were recorded with a Bruker AM 250 instrument at 250 MHz for ^1H spectra. The spectra were obtained in $CDCl_3$ solutions with trimethylsilane as the reference. IR spectra were obtained by evaporation of the solvent from the sample used for NMR spectra by using the KBr technique. A Bruker 113v FT-IR instrument was used (range, 4,000 to 600 cm^{-1}).

RESULTS

Fusarochromanone was detected in all 12 samples of poultry feed analyzed by TLC at levels ranging from 4 to 59 μg/kg (Table 1). This is the first detection of fusarochromanone as a naturally occurring contaminant. Strains of *F. equiseti* isolated from Danish cereals and other commodities were able to produce considerable amounts of fusarochromanone under optimal environmental conditions in the laboratory with rice as the substrate at yields ranging from 57 to 1,435 mg/kg (Table 2). The highest-ranking Danish strain, 1523, produced almost as much fusarochromanone as the reference strain from Alaska. Although no fungi from poultry feed were available for investigation, it is obvious that the potential is high for fungal production of fusarochromanone in Danish plant products. It appears (Table 1) that exposure to fusarochromanone in the range of 30 to 60 μg/kg of feed during 3 to 4 weeks results in 28 to 56% prevalence rates of TD. This seems to indicate an extremely pronounced toxicity of fusarochromanone. No other mycotoxin would develop macroscropically visible lesions during a few weeks of exposure to such low levels.

TABLE 1. TD lesions in broiler chickens and fusarochromanone concentrations in corresponding feed samples

Age of chicken (days)	% Prevalence of TD (live and dead birds combined)	Fusarochromanone concn (μg/kg)
Farm A		
0		34
7	0	
11		46
17		18
24		4
25	56	
28		5
34		10
35	27	
38	55[a]	
Farm B		
0		30
4	0	
8		59
11	0	
18	28	13
25	13	14
32		22
37	11	20
39	20[b]	

[a] Rate based on examination of 266 chickens collected at slaughter.
[b] Rate based on examination of 95 chickens collected at slaughter.

Chemical confirmation. The previous published mass spectrum of fusarochromanone obtained from a run using direct

TABLE 2. Production of fusarochromanone in rice by *F. equiseti* strains

Strain no.	Source of strain	Mean (SD) fusarochromanone concn (mg/kg)
1523	Barley	1,434.5 (369.9)
1524	Barley	484.8 (21.2)
1525	Barley	109.3 (80.1)
1526	Barley	130.0 (38.0)
1527	Barley	412.3 (309.6)
1528	Malt barley	56.7 (12.0)
1529	Peas	236.9 (118.2)
1469	Soil[a]	1,842.2 (559.6)

[a] This reference strain was isolated from a soil sample collected in Alaska by C. J. Mirocha.

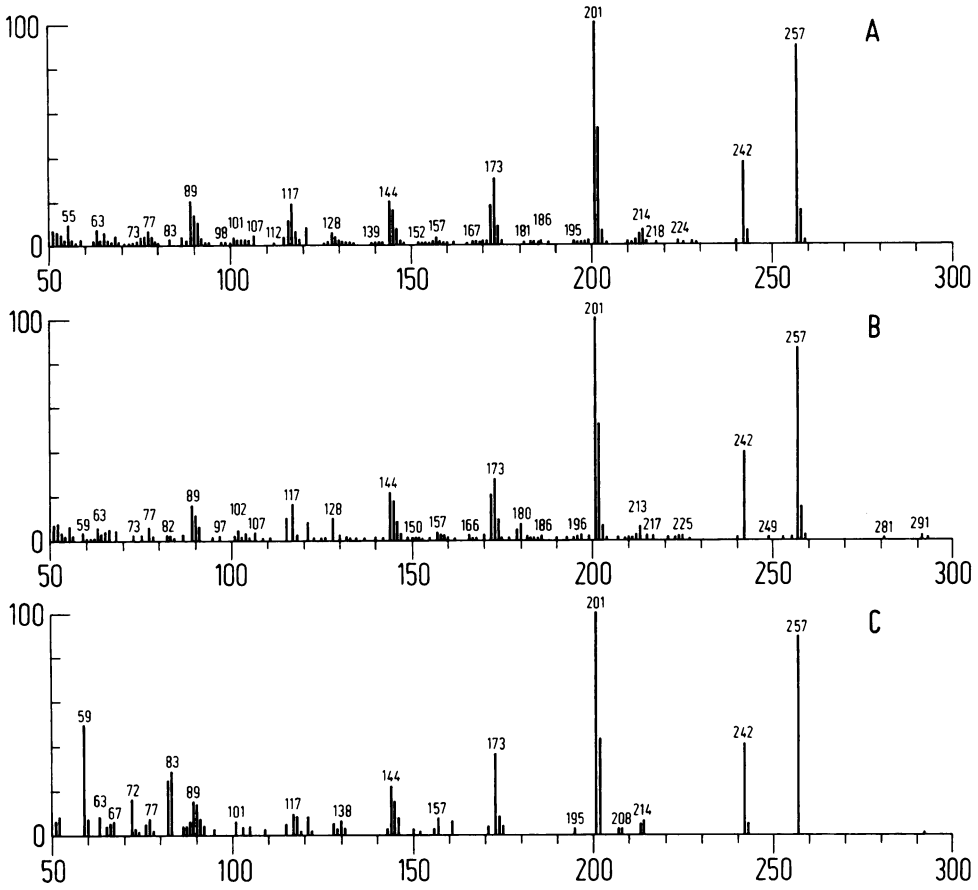

FIG. 1. (A) MS of fusarochromanone (approximately 1 μg/μl) introduced into the ion source through the gas chromatograph. (B) MS of sample I (scan 1055 from Fig. 2A). (C) MS of sample II (scan 1053 from Fig. 2C). Panels B and C are presented with the background subtracted.

inlet and electron impact (5) shows, in addition to a weak molecular ion at an *m/z* of 292, peaks at an *m/z* of 275 ($M^+ - OH$), an *m/z* of 257 ($M^+ - OH - H_2O$), an *m/z* of 244, and an *m/z* of 218 (base peak). This is in agreement with our results obtained by same technique. However, when fusarochromanone was introduced into the ion source through a gas chromatograph, the spectrum obtained (Fig. 1A) was completely devoid not only of the molecular ion but also of the peaks at *m/z*s of 275, 244, and 218. This can be ascribed to thermal degradation of fusarochromanone either in the injector or in the 300°C hot transfer line before introduction into the ion source. Investigations of the gas chromatographic-MS total ion current traces of samples I and II (Fig. 2A and C, respectively), therefore, concentrated on monitoring the ions at an *m/z* of 257. The corresponding traces are shown as inserts in Fig. 2B and D.

The MS data obtained from scans 1055 (sample I) and 1053 (sample II) are presented in Fig. 1B and C after subtraction of the background. Comparison of these spectra to the spectrum of an authentic sample of fusarochromanone run under the same conditions (Fig. 1A) left no doubt that fusarochromanone was present in samples I and II. To establish the presence of fusarochromanone firmly, sample I was injected into the gas chromatograph together with 3 μg of authentic material. This markedly increased the peak ascribed to the degradation product of fusarochromanone, while all other peaks in the gas chromatographic trace were unchanged.

Although sample I contained substantial amounts of impurities, the spectral region of 1700 to 1500 cm^{-1} showed some similarity to previously obtained IR spectra of fusarochromanone (S. V. Pathre, personal communication), indicating the presence of some chromanones. Peaks were observed at 1,658, 1,598, 1,565, and 1,550 cm^{-1}. The identification of the side chain (Fig. 3) was difficult because of strong absorption (impurities) in the NH and OH region (3500 to 3200 cm^{-1}), but comparison of the low-frequency part of the spectrum with that of authentic fusarochromanone (S. V. Pathre, personal communication) indicated the presence of chromanones other than fusarochromanone. The ^1H NMR spectra showed that the chromanone ring, but not the side chain in sample I, was intact, possibly because of great amounts of impurities.

DISCUSSION

The spectral data indicate that the compound detected and quantified by TLC in feed samples and rice cultures of *F. equiseti* strains was fusarochromanone with the structure suggested by Pathre et al. (7) (Fig. 3). Even the side chain data seem to be identical to previously published data, as revealed by the MS data, and the fact that no signals from the side chain were obtained by IR and NMR spectroscopy is most likely due to the small amount available for analysis. During analysis of feed samples and rice cultures, no attempts were made to detect TDP-2, the C-3′-*N*-acetyl deriv-

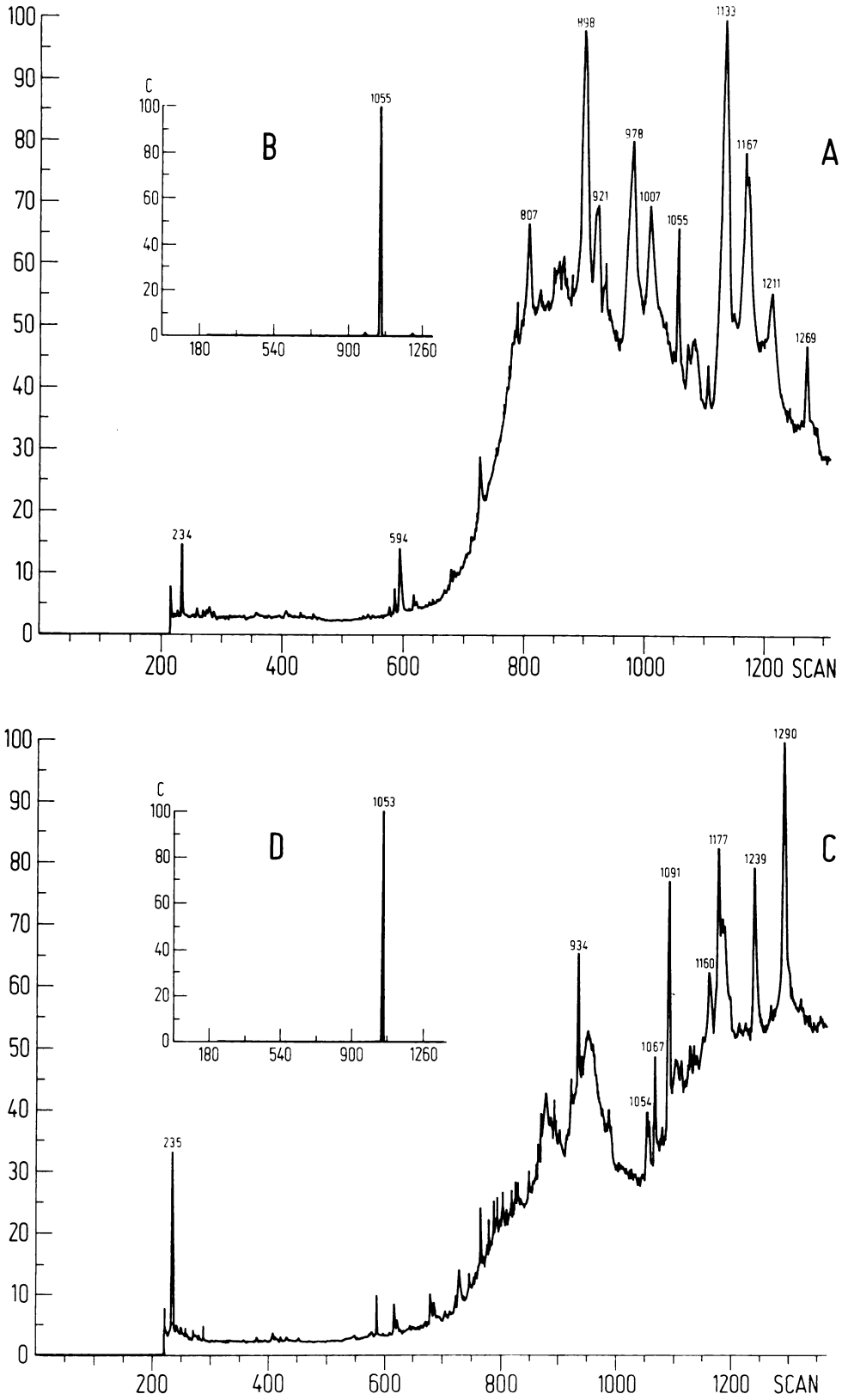

FIG. 2. Panels A and C are total ion current traces from injections of samples I and II, respectively, into the gas chromatograph. Monitoring the ion at an *m/z* of 257 gave traces B and D.

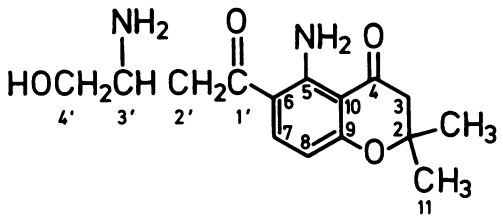

FIG. 3. Structure of fusarochromanone according to Pathre et al. (7).

ative of fusarochromanone, a metabolite found along with fusarochromanone in cultures of *F. equiseti* Alaska 2-2 grown under laboratory conditions (19). A comparison of the levels in feed (Table 1) and the yield of fusarochromanone by *F. equiseti* strains under optimal production conditions (Table 2) showed that the levels in feed were 10^3 to 10^4 times lower. This might indicate that only minute amounts of fungal cells in cereal feed are sufficient to produce fusarochromanone at microgram-per-kilogram levels. If this assumption is correct, one may expect fusarochromanone to be a widespread contaminant. No survey data are yet available, but we plan to screen cereal foods and feeds to assess the extent of fusarochromanone contamination.

In feeding experiments with pure fusarochromanone (labeled TDP-1), feed concentrations of 75 to 240 mg/kg induced TD and associated lesions in 80 to 100% of chickens during a 3-week exposure period (5, 17). The prevalence rate observed in the present study was considerably lower, but the exposure was also about 10^3 times lower. In one of the above-mentioned experimental studies (17), attempts were made to elucidate a possible interaction of selenium deficiency in the development of fusarochromanone-induced TD, but no interaction was observed. It is possible that the high fusarochromanone exposure conditions used overshadowed possible interactions. Thus, poultry experiments with lower exposure conditions, corresponding to the fusarochromanone concentrations in feed associated with field cases of TD, may more adequately elucidate possible interaction of other environmental factors on TD pathogenesis.

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