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TDP-1, a Toxic Component Causing Tibial Dyschondroplasia in Broiler Chickens, and Trichothecenes from *Fusarium roseum* 'Graminearum'†

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***Fusarium roseum* 'Graminearum' was isolated from overwintered oats in Alaska and was tested for its ability to cause tibial dyschondroplasia (TDP) in broiler chickens. The water-soluble fraction was tested and found to cause TDP. In addition, diacetoxyscirpenol and 7-hydroxydiacetoxyscirpenol were identified in the acetonitrile fraction of the extracts and caused mild mouth lesions in chickens. Six major water-soluble components were purified by thin-layer chromatography and tested for toxicity to chick embryos. One of the six components, called TDP-1, was found to be lethal to chick embryos. There was a 100% incidence of TDP in chickens fed a diet containing 75 ppm (wt/wt) of pure TDP-1, thus establishing the cause and effect relationship between TDP and TDP-1. Analyses by thin-layer chromatography and mass spectrometry revealed that TDP-1 is polar and ninhydrin positive, exhibits fluorescence with UV irradiation, and is a nitrogen-containing component with an empirical formula of $C_{15}H_{20}N_2O_4$.**

Species of the genus *Fusarium* are worldwide in their distribution. They are saprophytes as soil inhabitants and parasites of cultivated plants. Some isolates of certain species are capable of producing mycotoxins.

Mycotoxicoses, including moldy corn toxicosis in the midwestern United States (5), alimentary toxic aleukia in the Soviet Union (8), fusario-toxicosis in Canada (14), and red mold disease in Japan (22), are associated with the ingestion of moldy cereals infected with trichothecene-producing *Fusarium* species.

Tibial dyschondroplasia (TDP) is a common bone deformation found in growing broiler chickens and turkeys throughout the world. The lesion is characterized by the presence of the cone of cartilage which extends distally from the proximal tibiotarsal physis (4, 7, 12, 19). This deformation is of economic significance to the poultry industry. Shane (15) has reviewed the problems of TDP in broiler chickens in a popular livestock industry. According to his review, TDP affects up to 5% of the poultries of commercial flocks.

A number of factors such as rapid weight gain, nutritional deficiencies, and genetic factors have been cited as causes of TDP in poultry (10, 11, 13). Previously, *Fusarium roseum*-contaminated corn was found to induce a high incidence of leg lesions in broiler chicks (16). These were described as varus and valgus deformities, but they were not characterized.

We isolated one isolate of *F. roseum* 'Graminearum' from overwintered oats in Alaska and found it to be toxic to chickens. Walser et al. (19) first described the development of a wide cartilaginous band extending distally from the proximal physis of the tibiotarsi of chicks receiving dietary levels of a crude culture of this fungus. Allen et al. (1) have found that the same isolate caused near zero hatchability of fertile eggs by feeding 3% of the culture in a diet to laying hens. The toxic culture did not affect egg production and egg weight, but it killed the chick embryo. These previous studies of chickens prompted our interest in toxic metabolites as a possible cause of TDP and reduced hatchability of chicken eggs.

In this study, we report certain trichothecenes produced by this fungus and a new mycotoxin, given the trivial name of TDP-1, which causes TDP in chickens.

(A preliminary report on a portion of this work was presented at the Annual Meeting of the American Phytopathological Society, Salt Lake City, Utah, August 1982 [Y.-W. Lee and C. J. Mirocha, Phytopathology **72**:972, 1982]).

MATERIALS AND METHODS

Isolation and culture of fungus. The isolate of *F. roseum* 'Graminearum' (Corda) Sacc. emend. Snyder and Hansen was isolated from overwintered oats in Fairbanks, Alaska, in 1978. Stock cultures of the isolate were maintained on moist autoclaved soil and stored at −15°C. Inoculum was obtained by adding a few soil particles from the stock soil cultures to petri dishes of potato glucose agar. The agar plates were incubated for 5 days at 22 to 26°C.

Erlenmeyer flasks (1 liter) containing 200 g of Uncle Ben's converted long-grain enriched parboiled rice (Uncle Ben's Inc., Houston, Tex.) and 120 ml of distilled water were autoclaved for 1 h at 121°C on 2 consecutive days. The rice was then inoculated with mycelium plugs from 5-day-old potato glucose agar cultures. The flasks were hand shaken daily for the first week to provide uniform growth of mycelium throughout the substrate. After the cultures were incubated for 4 weeks, they were harvested, dried, and maintained at −15°C until use.

Preparation of culture extracts. Dried crude culture (750 g) was extracted with acetonitrile (1 liter, three times) and

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vacuum filtered through Whatman no. 4 filter paper. The filtrate was partitioned with petroleum ether (boiling point, 60 to 70°C) and concentrated in vacuo. The petroleum ether layer was discarded. This crude extract was dissolved in 100 ml of methanol, and 500 ml of distilled water was added. The methanol-water azeotrope was concentrated to about 200 ml. The water phase was applied to a column (3.5 by 60 cm) packed with washed Amberlite XAD-2 (150 g, 20-50 mesh; Mallinckrodt, Paris, Ky.). XAD-2 resin was activated by extraction with acetone overnight and rinsing with distilled water before use. The column was rinsed with 1 liter of distilled water and eluted with 1 liter of aqueous 90% methanol. The methanol eluate was concentrated to dryness in vacuo, dissolved in 50 ml of chloroform-methanol (9:1 [vol/vol]), and applied to a Florisil column (3.5 by 60 cm, 60-100 mesh; Fisher Scientific Co., Pittsburgh, Pa.), which was topped with 50 g of anhydrous sodium sulfate. The column was eluted with 1 liter of chloroform-methanol (9:1 [vol/vol]), and the eluate was called fraction I.

The residue remaining from the acetonitrile extract was reextracted with distilled water (2 liters, five times). The water extracts were pooled and applied directly to a column (3.5 by 60 cm) packed with previously washed XAD-2 resin as described above for the preparation of fraction I. The column was washed with 1 liter of distilled water, eluted with 1 liter of 90% aqueous methanol, and called fraction II.

Extraction and purification of TDP-1. Dried crude culture (500 g) was extracted with chloroform-methanol-ammonium hydroxide (90:10:1 [vol/vol/vol]; 2 liters, five times) and vacuum filtered. The filtrates were pooled, concentrated in vacuo to dryness, and dissolved in 200 ml of distilled water. The water phase was applied directly to a column (3.5 by 60 cm) packed with Amberlite XAD-2 resin as described for the previous extraction. The column was rinsed with 1 liter of distilled water and eluted with 1 liter of 90% aqueous methanol. The methanol eluate was concentrated in vacuo, dissolved in 50 ml of methanol, and mixed with 50 g of anhydrous sodium sulfate. After the powder was dried at room temperature, it was packed on the top of a column (3.5 by 60 cm) containing 150 g of Florisil. Successive elution was carried out with 2 liters of chloroform-methanol (9:1 [vol/vol]) followed by 2 liters of chloroform-methanol-ammonium hydroxide (90:10:1 [vol/vol/vol]). The TDP-1 fraction was detected by irradiating the column with long-wavelength UV light (365 nm) because TDP-1 produces an intense blue fluorescence under UV light. The TDP-1 fraction was dried in vacuo, dissolved in 50 ml of methylene chloride, and evaporated under nitrogen to yield a precipitate of TDP-1. The final preparation of TDP-1 displayed only a single spot on thin-layer chromatography (TLC) when resolved in the solvent system of chloroform-methanol-ammonium hydroxide (80:20:2 [vol/vol/vol]).

Chicken feeding tests for TDP. (i) Chick feeding test 1. A detailed description of the chick feeding test has been provided by Walser et al. (19). One-day-old broiler chicks (Arbor Acres-Ross Cross; Jack Frost, Inc., St. Cloud, Minn.) were maintained on the test diets for up to 3 weeks. Solutions of fraction I and fraction II were added to 750 g of ground corn and dried, and then each fraction was substituted into 10 kg of the basal corn diet for an equivalent amount of ground corn. The crude culture and residue remaining from the water extract were amended with the same balanced ration and adjusted to a concentration of 7.5%, respectively. Control chicks received quantities of uncontaminated feed equal to those consumed by chicks on the 7.5% test diets. Each treatment consisted of 10 chicks. All surviving chicks were euthanized after 3 weeks of feeding, and the incidence of TDP signs was recorded.

(ii) Chick feeding test 2. Pure TDP-1 (750 mg) dissolved in methanol was mixed with 500 g of ground corn and amended into 9.5 kg of the basal corn diet. The crude culture was mixed with the basal diet at a level of 5%. Each treatment consisted of 10 chicks. After a 3-week feeding period, all surviving chicks were euthanized, and the incidence of TDP signs was recorded.

Chick embryo test of water-soluble components in fraction II. Six major culture components were purified on preparative thin-layer chromatographic plates with a developing solvent system of ethanol-water (8:2 [vol/vol]). Each component was dissolved in dimethyl sulfoxide (Grade 1; Sigma Chemical Co., St. Louis, Mo.), and the concentration of each component was adjusted to ca. 1 μg/μl. The eggs were obtained from inbred, single-comb, White Leghorn female chickens crossed with Rhode Island red male chickens and were produced in the Department of Animal Science at the University of Minnesota. Fertilized eggs were set in a forced draft incubator at 39°C and 50 to 60% relative humidity. The eggs were candled on day 7 of incubation when the blood vessels of the chick embryos were clearly visible. Visible shell membranes of eggs were removed with forceps, and 20 μl of each component in dimethyl sulfoxide was dispersed onto the egg membrane. The holes in the shell were sealed with paraffin immediately after injection. The eggs were candled on days 4, 8, and 15 after injection, and mortality of embryos was recorded.

TLC. Fraction I was analyzed for trichothecenes with silica gel 60 plates (0.25-mm thick; E. Merck, Darmstadt, Federal Republic of Germany) with the developing solvent system of chloroform-methanol (9:1 [vol/vol]). The reagents used to detect trichothecenes on thin-layer chromatographic plates were 20% sulfuric acid in methanol, *p*-anisaldehyde solution, and reagents described by Kato and Takitani (9), which gave trichothecenes a blue color.

Fraction II was analyzed on high-performance silica gel thin-layer chromatographic plates (0.2-mm thickness, 10 by 10 cm; Whatman Chemical Separation, Inc., Clifton, N.J.) with developing solvent systems of ethanol-water (8:2 [vol/vol]) or chloroform-methanol-ammonium hydroxide (80:20:2 [vol/vol/vol]). Ninhydrin reagent solution and 20% sulfuric acid in methanol were used for detecting water-soluble components. Preparative TLC was accomplished with silica gel 60 plates (2-mm thick, 20 by 20 cm) to purify water-soluble components in fraction II. The developing solvent system was ethanol-water (8:2 [vol/vol]).

Spectroscopy. Low-resolution mass spectra were taken on a Hewlett-Packard 5987B gas chromatograph-mass spectrometer (GC-MS) for identification of trichothecenes. Positive chemical ionization (PCI) of trichothecenes was performed with methane as the reagent gas.

Low- and high-resolution mass spectra of TDP-1 were taken by direct inlet on an LKB-9000 GC-MS and an AEI MS 30, respectively.

A Finnigan 4535/TSQ triple quadruple MS equipped with positive-negative ion chemical ionization (CI) was used for CI of TDP-1 with ammonia.

The proton nuclear magnetic resonance (NMR) spectrum of 7-hydroxydiacetoxyscirpenol (7-OH-DAS) was obtained on a Varian XL-200 NMR spectrometer and was recorded in $CDCl_3$ solution.

Derivatization of metabolites. Samples for analysis of trichothecenes were reacted with trifluoroacetic anhydride (Pierce Chemical Co., Rockford, Ill.) to form trifluoroacetate

TABLE 1. Incidence of TDP by feeding culture extracts, residue, and 7.5% crude culture of *F. roseum* 'Graminearum' to broiler chickens

Treatment	No. dead/total no. treated	Incidence of TDP/ total no. examined
Control (basal diet)	0/10	0/10
Basal diet + fraction I	0/10	0/10
Basal diet + fraction II	2/10[b]	5/8
Basal diet + residue	1/10	8/10
Basal diet + 7.5% crude culture	9/10[c]	3/5

[a] Ten 1-day-old broiler chicks were fed test diets for 3 weeks.
[b] Of 10 chicks, 2 died within 1 week after feeding and were not examined for signs of TDP.
[c] Of 10 chicks, 5 died within 1 week after feeding and were not examined for signs of TCP.

TABLE 3. Time distribution of chick embryo mortality of fertile eggs after injection of six components in fractions II[a]

Treatment	Mortality (%) for the following incubation periods (days)[b]:		
	0–4	5–8	9–15
Control	10.0	0.0	0.0
NPS-1	0.0	0.0	10.0
NPS-2	0.0	0.0	5.0
TDP-1	90.0	0.0	10.0
NPS-3	0.0	0.0	10.0
TDP-2	0.0	5.0	0.0
TDP-3	0.0	0.0	10.0

[a] Each treatment replicated 20 eggs.
[b] Mortality was recorded at days 4, 8, and 15 days after injection.

derivatives. After the sample and trifluoroacetic anhydride were mixed, the solution was heated for 30 min at 45°C, the solution was dried under nitrogen, and 50 μl of toluene was added before the solution was injected into the GC-MS.

RESULTS

Incidence of TDP by feeding culture extracts to broiler chicks. The incidence of TDP brought about by feeding culture extracts, residue, and crude culture to broiler chicks is shown in Table 1. Fraction I did not cause mortality and signs of TDP, but it caused mild mouth lesions in 2 of 10 chicks. Mortality of chicks fed fraction II, residue, and 7.5% crude culture was 20, 10, and 90%, respectively, during a 3-week experimental period. The incidence of TDP in chicks fed fraction II, residue, and 7.5% crude culture was 63, 80, and 60%, respectively. Two chicks fed fraction II and five chicks fed the crude culture died within the first week after feeding, and these chicks were not tested for signs of TDP. The residue remaining from the water extract was toxic and caused mortality as well as signs of TDP in chicks.

Identification of trichothecenes in fraction I. When fraction I was chromatographed on thin-layer chromatographic plates and developed in chloroform-methanol (9:1 [vol/vol]), there was considerable streaking and nonspecific fluorescence which prevented identification of any thrichothecene. Fraction I was concentrated and applied to a column (1.5 by 12 cm) packed with C_{18} reverse phase silica gel. The column was eluted with a step gradient with water; 20, 50, and 70% methanol in water; and 100% methanol. Portions of the individual C_{18} eluates (reverse phase C_{18} column) were chromatographed on the plates with various trichothecene standards.

Resolution of C_{18} eluates by TLC with the chloroform-methanol (9:1 [vol/vol]) solvent system and the use of three spray reagents revealed two trichothecenes in the 50% methanol eluate. The first trichothecene showed the same R_f value (0.67) as that of the standard of diacetoxyscirpenol (DAS), and the second trichothecene with an R_f value of 0.56 remained unknown. These trichothecenes were confirmed by the rat skin test.

Bands corresponding to DAS and the unknown trichothecene were individually scraped and eluted. Gas chromatographic-mass spectrometric and NMR analyses of two trichothecenes revealed that one trichothecene (R_f = 0.67) was DAS and the other (R_f = 0.56) was 7-OH-DAS.

Analysis by TLC of water-soluble components in fraction II. Resolution of the water-soluble components by developing the plates in either the ethanol-water (8:2 [vol/vol]) or the chloroform-methanol-ammonium hydroxide (80:20:2 [vol/vol/vol]) solvent systems revealed that there were six major components in fraction II. Separation on high-performance silica gel thin-layer chromatographic plates for the resolution of water-soluble components are partially characterized in Table 2.

Three components were first noticed because of their fluorescent properties when irradiated with UV light. They fluoresced brightly when exposed to long-wavelength UV irradiation (365 nm) and less intensely when exposed to short-wavelength UV irradiation (260 nm). These three fluorescent components were named TDP-1 through TDP-3. Three other nonfluorescent components appeared to be gray spots after they were sprayed with sulfuric acid on TLC

TABLE 2. Characterization of six major water-soluble components of *F. roseum* 'Graminearum' by high-performance thin-layer chromatography

Compound[a]	Fluorescence under UV (365 nm)	R_f		Color reaction	
		Ethanol-water (80:20)	Chloroform-methanol-ammonium hydroxide (80:20:2)	20% sulfuric acid in methanol	Ninhydrin
NPS-1	Nonfluorescence	0.0	0.0	Gray	Pink
NPS-2	Nonfluorescence	0.11	0.01	Gray	Pink
TDP-1	Blue fluorescence	0.20	0.40	Dark brown	Pink
NPS-3	Nonfluorescence	0.60	0.57	Gray	Pink
TDP-2	Green fluorescence	0.80	0.62	Gray	None
TDP-3	Blue fluorescence	0.87	0.79	Brown	None

[a] TDP series components stand for fluorescent components with UV irradiation. NPS series components stand for ninhydrin-positive components which do not produce fluorescence with UV irradiation.

TABLE 4. Incidence of TDP by feeding TDP-1 and 5% crude culture of *F. roseum* 'Graminearum' to broiler chickens[1]

Treatment	No. dead/total no. treated	Incidence of TDP/ total no. treated
Control (basal diet)	0/10	2/10
Basal + 75 ppm TDP-1	1/10[b]	9/9
Basal + 5% crude culture	0/10	10/10

[a] Ten 1-day-old broiler chicks were fed test diets for 3 weeks.
[b] Of 10 chicks, 1 died within 1 week after feeding and was not examined for signs of TDP.

plates, and then the plates were charred at 110°C for 5 min. These three nonfluorescent components were named as NPS-1 through NPS-3. All NPS series components and only TDP-1 of the TDP series were ninhydrin positive, giving a pink color after ninhydrin solution was sprayed on the TLC plates.

Chick embryo test of water-soluble components in fraction II. Six major water-soluble components purified on TLC plates were subjected for toxicity evaluation in chick embryos. Twenty micrograms of TDP-1 per egg killed 100% of the chick embryos. The other five components did not affect the mortality of chick embryos (Table 3). Interestingly, 90% of chick embryo mortality occurred within the first 4 days of incubation after the injection of TDP-1.

Incidence of TDP in broiler chicks fed pure TDP-1. TDP was induced in chickens by feeding them pure TDP-1 (purity, 90 to 95% based on analysis by TLC); a 5% crude culture was used as a positive control (Table 4). Incidence of TDP signs resulting from both treatments were 100%, whereas 20% of the chicks showed TDP signs in the control. We have no explanation for the incidence in a basal diet control. Of the 10 chicks fed TDP-1, 1 died within 1 week after feeding; no mortality occurred in the chicks fed 5% crude culture.

Characterization of TDP-1 by mass spectrometry. The direct inlet mass spectrum of TDP-1 at 70 eV gave a base peak at *m/z* 218 and the largest fragment ion with an interpretable mass at *m/z* 292. The other major fragments were *m/z* 275, 257, 188, 162, and 106 (Fig. 1A). High-resolution mass spectrum of TDP-1 gave the largest ion at *m/z* 292 (calculated mass, 292.0422) and its empirical formula was $C_{15}H_{20}N_2O_4$.

The positive CI mass spectrum of TDP-1 in ammonia was obtained on a triple stage quadruple MS (Fig. 1B). Although the molecular ion is 292, an *m/z* of 585 was obtained, indicating that in the gas phase, TDP-1 can exist as a dimer. No fragmentation of fragment 585 was observed until the monomer 292 was obtained. The 585 dimer was also observed by fast atom bombardment ionization. Both fast atom bombardment and the triple stage quadruple subject the molecule to soft ionization (20 eV), and this may explain the lack of fragmentation between 585 and 293 (Fig. 1B).

DISCUSSION

In preliminary experiments, the crude culture was extracted with organic solvents such as ethyl acetate,

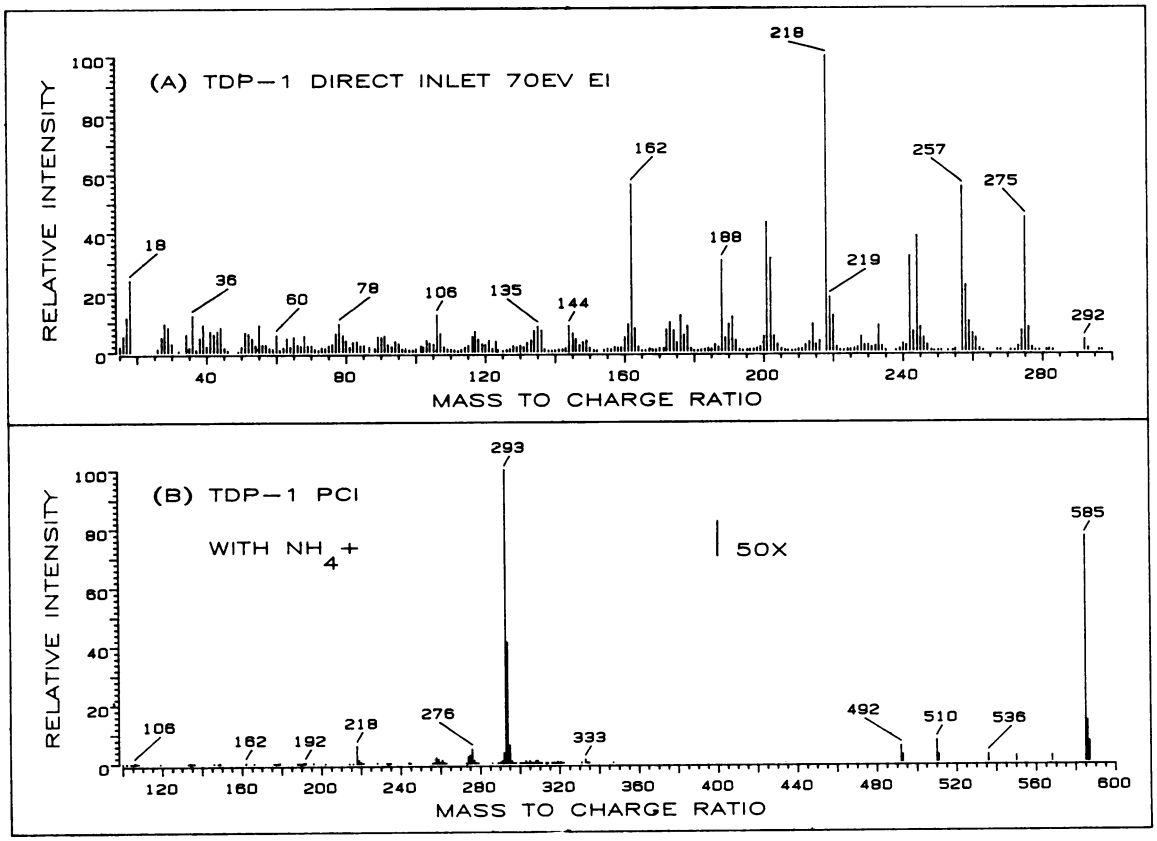

FIG. 1. (A) Full electron impact (EI) mass spectrum of TDP-1 by direct inlet. The molecular ion in the EI spectrum appears at *m/z* 292, and a base peak appears at *m/z* 218. (B) Full mass spectrum of TDP-1 in PCI with ammonia.

acetonitrile, and methanol, and then these crude extracts were tested for signs of TDP by feeding them to 1-day-old broiler chicks for 3 weeks. The crude extracts caused neither signs of TDP nor other toxic effects, except some mild mouth lesions in the chicks. However, residues remaining after extraction consistently caused mortality and signs of TDP in chick feeding tests. These results led us to conclude that the major toxic principle in *F. roseum* 'Graminearum' is water extractable and is neither a trichothecene nor a zearalenone.

In the first chick feeding test, mortality and incidence of TDP in chicks fed residues indicated that the toxins still remained in the residues after they were extracted five times with water.

The chicks fed fraction II and 7.5% crude cultures died within the first week after feeding, which did not allow them sufficient time to develop signs of TDP because this process takes 8 to 11 days (19). The kidneys of the chicks that died given test diets containing fraction II and crude culture were pale and enlarged, and the accumulation of white urates was frequently seen in the ureters.

DAS and 7-OH-DAS were identified in fraction I, which caused mild mouth lesions in only 2 of the 10 chicks and did not affect mortality. 7-OH-DAS has been reported first by Ishii (6), and its mean lethal dose in mice (intraperitoneal administration) is 3.5 mg/kg, making it more toxic than DAS (17). Quantitation by gas chromatography-mass spectrometry revealed that 12 ppm (wt/wt) of DAS and 25 ppm (wt/wt) of 7-OH-DAS were produced by this fungus based on DAS standard. These trichothecenes seem to be minor toxic components of *F. roseum* 'Graminearum.' Results of the comparison of the *F. roseum* 'Graminearum' culture and DAS also suggested that DAS is only partially responsible for the reduced hatchability, with the remainder being due to unknown toxins (1).

Isolation of unknown fungal metabolites requires some type of biological assay to monitor the progress of the purification effort. The chick embryo test is frequently used in bioassays for mycotoxins (2, 3, 18). In our study the chick embryo test was also effective to screen a toxic metabolite from a water-soluble fraction because a 20-μg injection of TDP-1 per egg resulted in 100% mortality.

In the second chick feeding test, purified (98%) TDP-1 caused 100% incidence of TDP signs in chicks. This result established the cause and effect relationship between TDP-1, a mycotoxin from *F. roseum* 'Graminearum,' and the incidence of TDP signs in broiler chickens, although many factors have been claimed to cause TDP in poultry. Thus, Koch's postulates as applied to mycotoxin research were satisfied.

Yang et al. (20, 21) have reported the correlation of the disease called Kaschin-Beck disease with the presence of *Fusarium* species in cereal grains produced in endemic areas. Young dogs and rats fed cultures of *Fusarium oxysporum* gave pathological and biochemical changes similar to those exhibited by humans with Kaschin-Beck disease; the necrotic tissues of bones could be reproduced by feeding *F. oxysporum* cultures to dogs. The chemistry of the toxin responsible for Kaschin-Beck disease is unknown, although it has been thought to be a nitrogen-containing compound (21). It is possible that TDP-1 may be involved in the disease as described in China.

We believe that TDP-1 is involved in the reduced hatchability of fertile eggs. The purified water-soluble fraction containing TDP-1 caused nearly zero hatchability of fertile eggs when it was fed to laying hens. Major mortality of embryos occurred during the 5 days of incubation of eggs (Y. W. Lee, C. J. Mirocha, D. J. Schroeder, and M. L. Hamre, Poultry Sci., in press).

The dimerization of TDP-1 observed in the ammonia CI mass spectrum seems unusual. We believe that the strong intermolecular hydrogen bonding of TDP-1 resulted in dimerization which gave a peak at *m/z* 585 in the CI spectrum. More recent information on the structure of TDP-1 based on mass spectroscopy, NMR, infrared spectroscopy, and X-ray crystallography have led to the postulation of the chemical structure. This paper will be published shortly.

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