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Citreoviridin Levels in *Eupenicillium ochrosalmoneum*-Infested Maize Kernels at Harvest

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Citreoviridin contents were measured in eight bulk samples of maize kernels collected from eight fields immediately following harvest in southern Georgia. Citreoviridin contamination in six of the bulk samples ranged from 19 to 2,790 μg/kg. In hand-picked samples the toxin was concentrated in a few kernels (pick-outs), the contents of which were stained a bright lemon yellow (range, 53,800 to 759,900 μg/kg). The citreoviridin-producing fungus *Eupenicillium ochrosalmoneum* Scott & Stolk was isolated from each of these pick-out kernels. Citreoviridin was not detected in bulk samples from two of the fields. Aflatoxins were also present in all of the bulk samples (total aflatoxin B_1 and B_2; range, 7 to 360 μg/kg), including those not containing citreoviridin. In Biotron-grown maize ears that were inoculated with *E. ochrosalmoneum* through a wound made with a toothpick, citreoviridin was concentrated primarily in the wounded and fungus-rotted kernels (range, 142,000 to 2,780,000 μg/kg). Samples of uninjured kernels immediately adjacent to the wounded kernel (first circle) had less than 4,000 μg of citreoviridin per kg, while the mean concentration of toxin in kernel samples representing the next row removed (second circle) and all remaining kernels from the ear was less than 45 μg/kg. Animal toxicosis has not been linked to citreoviridin-contaminated maize.

The discovery that *Eupenicillium ochrosalmoneum* Scott & Stolk infests preharvest maize and contaminates the damaged kernels with the neurotoxic mycotoxin citreoviridin (7, 8) required that we learn more about the distribution and amounts of the toxin in maize ears. Citreoviridin is thought to be the toxin responsible for the disease acute cardiac beriberi that has prevailed in Japan and Asia and that is associated with the consumption of molded and yellowed rice (6). There are no reported animal mycotoxicoses linked to the consumption of citreoviridin-contaminated maize. However, maize meal inoculated with citreoviridin-producing strains of *Penicillium pulvillorum* Turfitt and fed to day-old ducklings caused acute poisoning of these test animals (5). The development of an accurate quantitative method for determining levels of citreoviridin in maize kernels (R. D. Stubblefield, J. I. Greer, and O. L. Shotwell, J. Assoc. Off. Anal. Chem., in press) enabled us to analyze naturally contaminated maize kernels for citreoviridin. In addition, we examined the distribution of citreoviridin in maize ears that were produced in a controlled environment and wound inoculated with *E. ochrosalmoneum*.

MATERIALS AND METHODS

Citreoviridin in maize kernels at harvest. Eight bulk samples of maize kernels (2,000 g each) were collected immediately after combine harvesting of eight fields near Douglas, Ga., during the week of 12 to 19 September 1983. Moisture levels of the kernels in these samples ranged from 15.5 to 17%. A subsample (344 to 614 g) of each bulk sample was ground and analyzed for citreoviridin. A second set of subsamples (ca. 1,000 g) was examined for the presence of kernels or kernel fragments showing a bright lemon yellow pigment. Pigment-containing kernels (pick-outs) found in six of the subsamples were placed in tared vials and weighed prior to citreoviridin analysis. To determine whether the fungus producing the lemon yellow pigment was *E. ochrosalmoneum*, tiny fragments of fungus-infested endosperm were plated onto potato glucose agar. Samples (pick-outs and bulk) were then frozen (−7°C) until they could be analyzed for citreoviridin and aflatoxin.

Citreoviridin levels were determined by the method of Stubblefield et al. (in press). The maize was extracted with dichloromethane, and the extract was partially purified with silica and amino solid-phase extraction columns. The citreoviridin in the extract was quantitated by using normal-phase high-performance liquid chromatography. The mobile phase was ethyl acetate-hexane (75:25) at a flow rate of 1.5 ml/min. Fluorescence detection with 388-nm excitation and 480-nm emission gave an optimum response and sufficient sensitivity (limit of detection, 2 μg/kg) for use as an analytical method. The main peak, with a retention time of 5.46 min, was citreoviridin. Levels of aflatoxins in the ground bulk subsamples were determined by the method approved by the Association of Official Analytical Chemists for corn (26.028-26.058) (1).

Citreoviridin distribution in wound-inoculated maize ears. A loose-husked hybrid of maize used in the upper Midwestern corn belt (XL-12; DeKalb) was grown to maturity in a room with a controlled environment (photoperiod, 14 h light, 10 h dark; temperature, 30 ± 1°C during the day and 20 ± 1°C during the night; humidity, 82 ± 3%) in the Biotron facility (University of Wisconsin, Madison) (2). The controlled environment facility was used to regulate the physical environment of the developing maize ears while eliminating confounding variables such as kernel-contaminating fungi and damage by insects that infest maize.

A citreoviridin-producing strain of *E. ochrosalmoneum* (NRRL 6568) isolated from maize grown in Georgia (9) was used as the source of inoculum for this experiment. Conidia were harvested from 14-day-old Czapek agar slants that were incubated at 25°C. Five milliliters of 0.01% Triton X-100 was added to each slant, which was then agitated to suspend the conidia. The conidial suspensions were combined, filtered through glass wool, and adjusted to 10^6 spores

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TABLE 1. Citreoviridin and aflatoxin contamination in maize sampled at harvest (1981) from fields in southern Georgia

Bulk sample field	Bulk subsample A[a]			Yellow pick-out kernels[b]	
	Sample (g)	Aflatoxin B_1 and B_2 (μg/kg)	Citreoviridin (μg/kg)	Sample (g)	Citreoviridin concn (μg/kg)
1	546	50	ND[c]		
2	593	9	ND		
3	510	73	ND	0.22	53,800
4	579	7	24	0.27	689,900
5	508	310	19	0.100	479,100
6	580	360	810	0.26	759,900
7	614	120	2,510	1.24	656,300
8	344	250	2,790	4.32	484,700

[a] Analyses were performed without removal of lemon yellow pick-out kernels.
[b] Removed from 1,000-g subsample B.
[c] ND, Not detected (limit of detection, 2 μg/kg).

per ml with distilled water. The conidial suspension was stored at 4°C between applications.

To inoculate each ear, a sterile toothpick was inserted through the husk, removed, dipped into the spore suspension, and reinserted into the wound. Wound inoculations were performed on each of four ears at 7, 14, and 21 days after silking. Individual ears were inoculated with two opposing vertical rows of five toothpicks spaced 3.8 cm apart.

Kernels were removed from dried ears at harvest and segregated as follows: (i) wound-inoculated kernels; (ii) first circle, the four kernels immediately adjacent to a wound-inoculated kernel; (iii) second circle, those nonwounded kernels immediately surrounding the first circle; and (iv) all other kernels, representing the remaining kernels on the ear.

Kernels were surface sterilized in 2% sodium hypochlorite for 1 min, washed twice in sterile water, placed on malt extract agar in petri dishes (five kernels per plate), and incubated for 6 days at 25°C. The occurrence of *E. ochrosalmoneum* growing out from the kernels was recorded. Only the uninjured kernels from wound-inoculated ears were plated, since *E. ochrosalmoneum* consistently showed visible sporulation on lemon yellow-pigmented kernels that were wound inoculated with this fungus. All kernel samples were analyzed for citreoviridin by the method described above.

RESULTS AND DISCUSSION

The citreoviridin content of bulk samples from six fields ranged from 19 to 2,790 μg/kg (Table 1). The toxin was concentrated in a few kernels (pick-outs; range, 53,800 to 759,900 μg/kg), the internal tissues of which were stained a bright lemon yellow. Aflatoxins were detected in all eight of the bulk samples we examined (total aflatoxin B_1 and B_2; range, 7 to 360 μg/kg). Citreoviridin was not detected in bulk samples from two of the fields. The citreoviridin-producing fungus *E. ochrosalmoneum* and the aflatoxin-producing fungus *Aspergillus flavus* were isolated from all fragments of lemon yellow, citreoviridin-contaminated kernels that were plated onto potato glucose agar.

E. ochrosalmoneum infected nonwounded maize kernels from ears that were toothpick wound inoculated in the Biotron facility (Table 2). The number of infected kernels in samples representing the first circle, the second circle, and all other kernels varied considerably among individual ears. However, within a given treatment (e.g., inoculation at 7, 14, or 21 days following silking) the mean values for kernel infection were highest in kernel samples from the first circle surrounding the wound and lowest in kernel samples representing all other kernels from wound-inoculated ears.

TABLE 2. Infection of wound-inoculated maize ears with *E. ochrosalmoneum* and levels of citreoviridin accumulation

Postsilk day inoculated	Sample classification	Range (% [mean]) of kernel infection	Mean sample wt (g)	Citreoviridin concn (μg/kg)	
				Range	Mean
7	Wounded kernels		0.1	142,000–910,000	554,000
	First circle	47–80 (62)	8.7	ND[a]–980	240
	Second circle	8–96 (53)	19.5	ND	ND
	All other kernels	0–94 (44)	104.6	ND	ND
14	Wounded kernels		1.3	1,200,000–1,966,000	1,544,000
	First circle	7–47 (22)	11.2	ND–3,920	1,000
	Second circle	0–64 (19)	19.0	ND–42	14
	All other kernels	2–34 (12)	100.3	ND	ND
21	Wounded kernels		1.1	1,246,000–2,780,000	2,135,000
	First circle	67–87 (75)	12.2	ND–1,370	420
	Second circle	20–80 (52)	16.7	ND–8	3
	All other kernels	14–54 (40)	71.2	ND–5	1

[a] ND, Not detected (limit of detection; 2 μg/kg).

The distribution of citreoviridin was limited primarily to the wounded and fungus-infested kernels (range, 142,000 to 2,780,000 μg/kg). Samples of uninjured kernels immediately adjacent to the wounded kernels (first circle) had levels of citreoviridin contamination of less than 4,000 μg/kg, while mean amounts of toxin in kernel samples representing the next row removed (second circle) and all remaining kernels from the ear were less than 45 μg/kg. Wicklow and Cole (7) found citreoviridin in insect-damaged and mold-infested kernels, with bright lemon yellow endosperm tissues, from standing maize that was left unharvested in a south Georgia field. Our results indicated that citreoviridin contamination of wound-inoculated maize ears is localized primarily in wounded kernels. For example, mean concentrations of citreoviridin in samples of uninjured kernels in the first circle surrounding individual wounded kernels were 1,500 to 5,000 times lower than those in the wounded kernel tissues. By contrast, mean concentrations of aflatoxins in equivalent samples of the uninjured surrounding kernels from ears of the same hybrid that were similarly toothpick wound inoculated with *A. flavus* 21 days following silking were only 16 to 60 times lower than those in the wounded kernel tissues (D. T. Wicklow, B. W. Horn, O. L. Shotwell, C. W. Hesseltine, and R. W. Caldwell, Phytopathology, in press). *E. ochrosalmoneum*, like *A. flavus*, is weakly pathogenic to intact maize kernels, but can rapidly infest and rot damaged kernels and contaminate these kernels with substantial quantities of toxins.

The findings of this study indicate that an amount of toxin exceeding the 50% lethal dose for mice (i.e., 29 mg/kg, when administered orally) (6) may be present in less than 0.5 g of naturally fungus-rotted maize kernels. A single dose of purified citreoviridin near the 50% lethal dose for male mice induced symptoms such as lameness of the posterior extremities, impairment of voluntary movement, and tremors (6). Maize ears infested with *E. ochrosalmoneum* and contaminated with citreoviridin was associated with staggers in cattle that ate lodged corn (3). Lodged ears in south Georgia are often infested with *E. ochrosalmoneum*, and many of the kernels are contaminated with a lemon yellow pigment (D. T. Wicklow, personal observation). There is no information available on the toxicity of citreoviridin to cattle or any other ruminant animals, but sheep fed on chestnuts molded with *E. ochrosalmoneum* exhibited neurotoxic symptoms corresponding to those caused by citreoviridin (4).

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