Production of Penitrem A and of an Unidentified Toxin by Penicillium lanoso-coeruleum Isolated from Weevil-Damaged Pecans

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ABSTRACT

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Chloroform extracts of *Penicillium lanoso-coeruleum* isolated from weevil-damaged pecans and grown on shredded wheat-yeast extract-sucrose medium were toxic to 1-day-old cockerels, producing sustained tremors and convulsions prior to mortality. Two toxic fractions were separated from chloroform extracts by sequential elution from silica gel columns with diethyl ether and acetone. Physical and chemical characteristics of purified crystals from the ether fraction correlated with those of the mycotoxin Penitrem A: an R_f value in toluene:ethyl acetate:formic acid (5:4:1, v/v) (TEF) of 0.5; ultra-violet absorption maxima (UV $_{max}$) in methanol at 234, 283, 294, and 300 nm; and infrared (IR) absorptions also identical to those of authentic Penitrem A. Methanolysis resulted in a color reaction specific for Penitrem A. Crystals from the acetone fraction had R_f values in TEF of 0.1 or less, UV maxima at 317 and 245 nm, and IR maxima of 1,675 and 1,400 cm^{-1}. The chemical identity of the toxin in the acetone fraction is unknown.

Additional key words: mycotoxin, tremorgen.

One of the most serious pests of the pecan [*Carya illinoensis* (Wang) K. Koch] in the southeastern United States is the pecan weevil, *Curculio caryae* (Horn). Larvae hatched from eggs deposited in nuts early in the growing season consume a large portion of the kernel before emerging through the shell in September and October (3). Weevil infestations seriously reduce the yield and quality of marketable nuts. In a recent survey in Georgia, 8 of 160 pecan samples destined for shelling plants contained 3-5% weevil-damaged nuts, and aflatoxin contamination was associated with the infested samples (Wells, *unpublished*). In addition, a wide array of toxigenic fungi has been isolated from weevil-damaged pecans (9). Generally, a large percentage of, but not all, discolored and weevil-damaged kernels and pieces are hand-selected from shelling plant lines. Those that are overlooked do enter consumer food channels.

Penitrem A is a toxic fungal metabolite originally isolated from *Penicillium cyclopium* Westling by Wilson et al. (10) and from *P. palitans* Westling by Ciegler (1). The fungi were cultured from moldy feedstuffs suspected to be involved in the death of sheep, horses, and cattle. Clinical signs of toxicosis by purified toxin in laboratory mice (administered orally) were behavioral and neurological, including sustained tremors and convulsions prior to mortality. Penitrem A also has been isolated from *P. crustosum* Thom, and *P. puberulum* Bainier, both of which grow on various agricultural commodities (7). Ciegler and Pitt (2) observed that production of Penitrem A by members of the genus *Penicillium* is confined to the subsection Fasciculata (Asymmetrica). Most ioslates that produce Penitrem A also produce Penitrem B and Penitrem C (6).

Penicillium lanoso-coeruleum Thom was one of the toxigenic species of *Penicillium* isolated by Wells and Payne (9) from pecans damaged by the pecan weevil. Toxicity of *P. lanoso-coeruleum* to 1-day-old cockerels was characterized by sustained tremors and convulsions prior to death.

This report presents evidence that the toxicity of *P. lanoso-coeruleum* is caused by two distinct toxins. One was identified as Penitrem A, and the other as a chemically distinct metabolite. To the authors' knowledge, this is the first report of Penitrem A production by *P. lanoso-coeruleum*.

MATERIALS AND METHODS

Bioassay for toxicity.—Cultures of toxin-producing *P. lanoso-coeruleum* were obtained from weevil-damaged pecans in a previous study (9). Isolates designated as P 23 (ATCC 32017), P 29 (ATCC 32018), P 39 (ATCC 32019), and P 89 (ATCC 32020) that had been maintained on potato-dextrose agar slants at 1 C were subcultured for the present investigation. Isolates then were cultured on shredded wheat-yeast extract-sucrose (YES) medium (8), and on autoclaved or fresh pecan media. Autoclaved pecan medium was prepared by autoclaving 50 g of shelled pecan halves and 5 ml water in 500-ml flasks for 15

min at one atmosphere gauge pressure. Fresh pecan medium was prepared with pecan halves surface-sterilized for 5 min with a 10% solution of commercial Clorox (0.5% sodium hypochlorite) and rinsed with sterile distilled water. About 50 g of sterilized pecan halves then were transferred aseptically to sterile 500-ml flasks. All media were inoculated by mass transfer of fungal cultures and incubated for 3 wk at 21 C. Cultures then were extracted with chloroform. The extracts were tested for toxicity by the method of Kirksey and Cole (8), except that cultures grown on pecan media and containing pecan oil did not require the addition of corn oil as an inert carrier.

The toxicity of extracts was determined by cockerel bioassay via crop intubation (8). Check animals were dosed with extracts of noninoculated shredded wheat or pecan media. Dosed cockerels were examined daily for clinical signs of toxicity, and survival ratios were calculated 5 days after dosing. Bioassays were repeated at least three times at approximately 3- to 6-mo intervals.

Production and isolation of toxin.—*Penicillium lanoso-coeruleum* (ATCC 32017) was cultured in 60 Fernbach flasks (2.8-liter) containing 200 g shredded wheat and 200 ml mycological broth (pH 4.8, Difco) amended with 1.6% yeast extract. After 4 wk of culture growth at 21 C, the mycelial mats were comminuted 1 min with 500 ml of chloroform in a Waring Blendor. Solid residue was separated from the chloroform extract by filtration with four layers of cheesecloth, and the extract was clarified by vacuum filtration through a 2-cm pad of anhydrous sodium sulfate on a glass fiber filter pad (Reeve-Angel). The mycelial residue was re-extracted with 300 ml of chloroform, and the clarified filtrates were combined. Chloroform was removed from filtrates by flash evaporation, and the resulting concentrate was fractionated on a silica gel column, 11 × 40-cm [420-246 μm (40-60 mesh)], by sequential elution with 3 liters each of hexane, diethyl ether, chloroform, ethyl acetate, acetone, and methanol. Fractions were collected in 500-ml aliquots and 50-ml samples from each were bioassayed for toxicity to 1-day-old cockerels (8). Toxic ether fractions were combined, concentrated, and re-chromatographed on 4.5 × 60-cm columns by gradient elution from pure hexane to pure diethyl ether, and the eluate was collected in 15-ml fractions. Toxic acetone fractions were similarly combined and re-chromatographed by gradient elution from pure ethyl acetate to pure acetone.

Presence of the toxins in eluted fractions was monitored by the cockerel bioassay. One-tenth of each 500-ml aliquot was concentrated, mixed with 3.5 ml of corn oil, and heated in a steam bath for 2 hr to evaporate the solvent. Cockerels were each dosed with 1 ml of corn oil-extract mixture. Every tenth tube of the rechromatographed fractions (15 ml) was concentrated similarly, mixed with corn oil, and bioassayed.

TABLE 1. Survival[a] and symptoms of 1-day-old cockerels dosed with chloroform extracts of four isolates of *Penicillium lanoso-coeruleum* grown on artificial and pecan media

Isolate	Shredded wheat-YES medium				Pecan medium			
	Survival for replication				Autoclaved halves		Fresh halves	
	1	2	3	Tremors[b]	Survival	Tremors	Survival	Tremors
P23	2/5	2/5	1/5	+	0/5	+	0/5	+
P29	4/5	0/5	1/5	+	1/5	+	2/5	+
P39	2/5	0/5	0/5	+	2/5	+	2/5	+
P89	1/5	0/5	0/5	+	0/5	+	0/5	+

[a]Number of surviving/number of cockerels 5 days after dosing by crop intubation. Control group survival was 100%.
[b]Sustained tremors and convulsions prior to death.

TABLE 2. Survival and clinical symptoms of 1-day-old cockerels dosed with diethyl ether and acetone fraction subsamples[a] of *Penicillium lanoso-coeruleum* toxins chromatographed on a silica gel column by sequential elution with six different solvents

Solvent	Fraction number	Survival at indicated days after dosing			Clinical symptoms
	(500 ml)	1	3	5	
Diethyl ether	1	3/3	0/3	–	tremors
	2	3/3	0/3	–	tremors
	3	3/3	0/3	–	tremors & convulsions
	4	3/3	0/3	–	tremors
	5	3/3	3/3	3/3	none
	6	3/3	3/3	3/3	none
Acetone	1	3/3	2/3	2/3	tremors
	2	3/3	0/3	–	tremors & convulsions
	3	3/3	0/3	–	tremors
	4	3/3	0/3	–	tremors
	5	3/3	2/3	2/3	none
	6	3/3	3/3	3/3	none

[a]A 50-ml aliquot of each fraction evaporated to dryness and redissolved in 3.5 ml of corn oil. Three 1-day-old cockerels per fraction were dosed by crop intubation with 1 ml of corn oil extract.

Toxins in the eluates also were monitored by silica gel (Mallinckrodt Silicar TLC-7G) thin-layer chromatography (TLC). The developing solvents were toluene:ethyl acetate:formic acid (5:4:1, v/v) (TEF) and chloroform:acetone (93.7, v/v). Spots were made visible by spraying developed plates with ethanolic H_2SO_4 and then heating them 2-4 min at 100 C. Toxic fractions were combined and evaporated in the dark at 1 C. The residues were recrystallized for physical and chemical analyses in redistilled solvents.

Physical and chemical properties of the toxins.—The presence of the toxins in extracts from inoculated, fresh pecans also was determined by TLC. Clean-up procedures for pecan oil in the crude extracts were those of Hagan and Tietjen (4). Colorimetric tests for Penitrem A and B were based on the methanolysis method of Hou et al. (5). Ultraviolet (UV) absorption spectra of purified fractions dissolved in MeOH were obtained with a Beckman DB-GT Spectrophotometer. Infrared (IR) spectra of samples as thin films on KBr blocks were obtained with a Perkin-Elmer Model 257 IR Spectrophotometer.

RESULTS

Biological properties of toxins.—The four isolates of *P. lanoso-coeruleum* tested were toxic to 1-day-old cockerels. Toxicity of isolates cultured on shredded wheat-YES medium and on fresh or autoclaved pecan halves was high, causing over 50% mortality (Table 1). Tremorgenic symptoms also were produced by all isolates tested, and on all dosed cockerels.

The production of toxins by our stocks of *P. lanoso-coeruleum* was sustained and undiminished during the period of this experimentation, and throughout 4 yr of periodic subculturing after original isolations were made.

Two distinct toxic fractions were separated by column chromatography from chloroform extracts of *P. lanoso-coeruleum*. One was eluted with pure diethyl ether and the other with pure acetone (Table 2). Intermediate fractions eluted with ethyl acetate or with chloroform were not toxic.

Toxicity of all active fractions was high, characterized by tremorgenic symptoms and by 100% mortality within 3 days of dosing. The most active fractions of each of the eluted toxins caused severe convulsions and loss of muscular control.

Physical and chemical properties.—Physical and chemical properties of the two toxins from *P. lanoso-coeruleum* were distinctly different. Methanolysis of the toxic ether fraction, but not of the toxic acetone fraction, produced a blue color reaction (Table 3). The positive reaction, a specific test for Penitrem A or B, was confirmed by comparison with an authentic sample of Penitrem A obtained from J. B. Wilson, Vanderbilt University, Nashville, Tennessee.

The R_f value on TEF (0.5) of the toxic ether fraction also correlated with that of authentic Penitrem A (Table 3). The toxic acetone fraction, however, had an R_f of 0.1 or less. Thin layer of chromatograms of the ether fraction gave dark blue or black spots, whereas that of the acetone fraction gave orange-red spots. Thin-layer chromatography of toxic crude extracts of infected, fresh pecans, processed for removal of lipids (4), yielded spots comparable to those of the toxic ether and the acetone fractions.

The UV absorption maxima of the toxin from the ether fraction were at 234, 283, 294, and 300 nm, and those of the acetone fraction were at 317 and 245 nm. Infrared absorption maxima of the two toxins differed also, with no peaks in common (Table 3). Ultraviolet and IR spectra of the toxin from the ether fraction were identical to those published for Penitrem A (6) and to those obtained with an authentic sample.

DISCUSSION

We conclude that the toxin isolated from the ether extract of *P. lanoso-coeruleum* is Penitrem A. The more polar compound, isolated from the acetone fraction, has not yet been identified and is currently under investigation.

Our finding that Penitrem A is produced by isolates of *P. lanoso-coeruleum* is consistent with the observation that those Penicillia taxonomically classified in the subsection Fasciculata can produce Penitrem A (2).

Although the toxins from *P. lanoso-coeruleum* were detected initially and isolated from cultures growing on an artificial medium, we present evidence that the organism can produce the toxins on pecans under laboratory conditions. The presence of the organism on pecans, as surface contaminants or as mycoflora established in infected tissues, suggests only that a potential problem may exist. Further research is needed

TABLE 3. Chemical and physical properties of the toxic diethyl ether and acetone fractions of crude extract of *Penicillium lanoso-coeruleum* eluted from a silica gel column, and of authentic Penitrem A

Property	*P. lanoso-coeruleum* fraction		Authentic Penitrem A
	Eiethyl ether	Acetone	
R_f value	0.5	0.1	0.5
TLC reaction[a]	dark blue	orange-red	dark blue
Color reaction[b]	blue	pale green	blue
UV absorption maxima[c]	234, 283, 294, and 300 nm	245, 317 nm	234, 283, 295, and 300 nm
IR absorption maxima[d]	1375, 1645, 3390, 3480 cm^{-1}	1675, 1400 cm^{-1}	1375, 1645, 3390, 3480 cm^{-1}

[a]Thin-layer chromatography plates developed with toluene:ethyl acetate:formic acid (5:4:1, v/v), sprayed with ethanolic H_2SO_4, and heated (2-4 min at 100 C).

[b]Methanolysis by the method of Hou et al. (5).

[c]In methanol.

[d]As a thin film on KBr block.

to determine whether these organisms are capable of growth and toxin production under natural conditions.

The potential for multiple toxin production by fungi on insect-damaged pecan tissues emphasizes the need for weevil-eradication programs in the orchards and for good quality control in the shelling plants.

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