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Isolation of Citreoviridin from *Penicillium charlesii* Cultures and Molded Pecan Fragments

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The extraction and systematic fractionation of *Penicillium charlesii* Smith cultures and contaminated pecan fragments yielded the yellow mycotoxin citreoviridin. Citreoviridin proved acutely toxic to 1-day-old chickens, with an oral 50% lethal dose of 37.5 mg/kg, and showed plant growth inhibition in wheat coleoptiles even at concentrations as low as 10^{-4} M. It was toxic to corn seedlings but did not affect young tobacco seedlings.

Production of nuts from pecan plants (*Carya illinoensi* (Wang) K. Koch) in the United States in 1979 amounted to 100,000,000 kilos (U.S. Department of Economic Statistics and Cooperative Services, 14 June 1980). An estimated 75% of cultured pecans are processed in commercial shelling plants to separate nutmeats from shells and integuments (22). Shell material constitutes half of the fresh weight of pecans and is generally used as landfill or is marketed as mulch for ornamental plants.

Waste shell material from pecan-processing plants contains small amounts of nutmeat fragments. Once shells and kernel fragments are placed outdoors and exposed to moist environmental conditions, they can support a rich variety of saprophytic microorganisms, including toxigenic species of fungi (J. M. Wells, unpublished data). When pecan shells are used as mulch for ornamental plants, the moldy fragments present in the shells are a food source for foraging wildlife. Thus, toxigenic metabolites produced by fungi on pecan wastes may be introduced into the wildlife environment and may enter the wildlife food chain.

There are a few reports of the natural occurrence of fungal metabolites in pecans. Schroeder and Cole (16) found two metabolites from *Alternaria* spp., alternariol monomethyl ether and alternariol, in pecan culls obtained from commercial pecan-shelling plants. Aflatoxin has been detected in samples of commercially grown pecans collected late in the growing season (21). However, most reports associating mycotoxins with pecans have been based on in vitro studies of the toxin-producing capabilities of those fungi isolated from pecans.

During January 1980 in southwest Georgia, we observed that a heap of pecan shells being used for plant mulch around a house contained very moldy kernel fragments. Birds of various species and native grey squirrels (*Sciurus carolinensis*) foraged for the moldy pecan fragments. We isolated the fungal species in pure culture and evaluated their toxigenic nature with experimental animals.

We now report the isolation of a highly toxigenic strain of *Penicillium charlesii* Smith and the natural occurrence of high levels of the mycotoxin citreoviridin in moldy pecan fragments.

MATERIALS AND METHODS

A representative sample of moldy pecan kernel fragments was handpicked from shells and debris. A portion of the sample was used to evaluate the nature of the fungal population. A total of 932 g of the moldy kernel fragments were frozen for future analysis.

Fungi were isolated from the moldy kernel fragments and from spore dust at the collection site with potato dextrose agar plates. *Penicillium* species were identified with the aid of Raper and Thom's *Manual of the Penicillia* (12) and the revision by Samson, Stolk, and Hadlock of Raper and Thom's subsection Fasciculata (15). Recently however, Pitt published a report on a new and comprehensive treatment of species of *Penicillium* and related genera with *Penicillium* anamorphs (11). We have indicated where Pitt differs from Raper and Thom and from Samson, Stolk, and Hadlock. Fungi were incubated at 25 to 27°C, and cultures were maintained at 5°C. To determine toxigenicity, we cultured each isolate for 2 weeks at 25 to 27°C on two different media; one contained 25 g of

shredded wheat supplemented with 50 ml of mycological broth (Soytone [10 g/liter]-dextrose [40 g/liter], adjusted to pH 4.8) plus 2% yeast extract-15% sucrose, and the other contained 25 g of rehydrated pecan meats (ca. 20% moisture) surface sterilized with 1% sodium hypochlorite. The toxigenicity of each isolate was determined by the method of Kirksey and Cole (6) with 1-day-old chickens dosed orally via crop intubation.

For mass production of the toxin, *P. charlesii* was cultured for 14 days at 25 to 27°C in 28 Fernbach flasks (2.8 liters), each containing 100 g of shredded wheat and 200 ml of broth, as described above.

Extraction and isolation of the toxin. Citreoviridin was extracted from the *P. charlesii* cultures by homogenization with hot $CHCl_3$ for 2 min with an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, Ohio). The extract was filtered through cheesecloth, dried over anhydrous sodium sulfate, and concentrated under vacuum at 60°C.

The toxic crude extract was fractionated on a Silica Gel 60 column (9.5 cm [inside diameter] by 17 cm) packed as a slurry in benzene. Purification of the toxin was monitored with 1-day-old chickens dosed orally via intubation. The column was eluted sequentially with 2 liters of benzene-3 liters of ethyl ether-2 liters of ethyl acetate-2 liters of acetone. Toxicity was associated exclusively with the ethyl acetate fraction. The ethyl acetate fraction was reduced in volume, and the toxin crystallized after 2 days at 5°C. The sample of moldy pecan kernels previously collected and frozen was processed in the same fashion.

Animal and plant bioassay. Crude extracts and pure toxin in corn oil suspension were administered via crop intubation to 1-day-old chickens at 1 ml per chicken. Weil's tables (20) were used for 50% lethal dose determinations after citreoviridin was administered to 10 chickens each at levels of 6.25, 12.5, 25, and 50 mg/kg.

In keeping with our policy of routinely screening animal toxins for plant hormone activity, citreoviridin was tested in plant systems.

Coleoptile sections 4 mm long were cut from 4-day-old wheat seedlings (*Triticum aestivum* L. cv. Wakeland) grown in the dark on moist trays of sand at 22 ± 1°C (5). They were incubated in buffered solutions (pH 5.6) containing 2% sucrose (10) and various concentrations of citreoviridin. Citreoviridin was dissolved in 75 μl of acetone and adjusted to 10 ml with buffer-sucrose solution to give a final concentration of 10^{-3} M (1). From this solution, serial dilutions were made to 10^{-4}, 10^{-5}, and 10^{-6} M, and 2 ml of each concentration was placed into test tubes with 10 coleoptile sections. After incubation for 24 h at 22°C in a roller tube appratus at 0.25 rpm, coleoptile sections were measured (3× images produced by a photographic enlarger), and data were statistically analyzed (7).

Citreoviridin was also tested on intact greenhouse-grown plants. Six-week-old tobacco seedlings (*Nicotiana tabacum* L. cv. Hicks) were sprayed with aqeuous solutions of the metabolite at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M plus 0.1% Tween 20-10% acetone. Individual plants were treated with 1 ml of test solution that contained 4,020, 402, or 40.2 μg of citreoviridin. Replication was sixfold, with observations made at 7 and 14 days after treatment.

Ten-day-old corn seedlings (*Zea mays* L. cv. Norfolk Market White) were treated by placing 100 μl each of 10^{-2}, 10^{-3}, and 10^{-4} M solutions of the aforementioned aqueous formulation into leaf whorls. Actual amounts of each solution were 402, 40.2, and 4.02 μg per whorl, respectively. There were four corn plants per pot, and each pot treatment was triplicated. Observations were made at 7 and 14 days after treatment.

Physical and chemical analyses. Thin-layer chromatography (TLC) analyses were performed on precoated Silica Gel 60 F-254 plates (5 by 10 cm; EM Laboratories, Inc., Elmsford, N.Y.) with a developing solvent system of toluene-ethyl acetate-formic acid (5:4:1; vol/vol/vol). Developed plates were examined in normal light and under long-wave ultraviolet light to visualize the toxin.

Ultraviolet and visible spectra of citreoviridin in methanol solution were recorded with a Beckman model DB-G recording spectrophotometer. Extinction coefficients at λ_{max} 802 and 390 nm were used to quantitate the citreoviridin content of the pecan fragments gathered from the pecan mulch. The ethyl acetate fraction from moldy pecan fragments was dissolved in 1 liter of methanol, and a 1:20 dilution of this solution was used for comparison with extinction coefficients of a 10^{-5} M solution of authentic citreoviridin and with extinction coefficients for citreoviridin reported at 390 nm and experimentally determined at 802 nm (9).

Infrared spectra of samples prepared as thin films on KBr windows were obtained with a Perkin Elmer model 257 recording spectrophotometer equipped with a 4× beam condenser. ^1H and ^{13}C nuclear magnetic resonance spectra were obtained with a Varian Associates XL-100-12 nuclear magnetic resonance spectrometer equipped with the 620-L disk data system. Samples were analyzed in d_6-acetone solution in 5-mm sample tubes, with tetramethylsilane as an internal reference. Spectra were run in the Fourier transform mode with 8 K data points, with a sweep width of 1,000 Hz for the ^1H spectra and 5,000 Hz for the ^{13}C spectra. Single-frequency, off-resonance, proton-decoupled ^{13}C spectra were performed to aid in distinguishing the types of carbon atoms.

Mass spectra were obtained with a VG micromass 70/70 spectrometer. Samples were introduced into the spectrometer via the direct probe technique.

RESULTS AND DISCUSSION

The microflora of these contaminated pecan shells and kernel fragments was apparently limited by ambient temperature to two fungal species, *Penicillium verrucosum* Dierckx var. *cyclopium* (Westling) (Samson et al. [15]) (= *Penicillium aurentiogriseum* Dierckx, according to Pitt [11]) and *P. charlesii* (considered a synonym of *Penicillium fellutanum* Biourge by Pitt [11]). Both fungi were toxigenic to orally dosed 1-day-old chickens when cultured on the shredded wheat medium. However, only the *P. charlesii* isolate proved to be toxic when grown in pure culture on surface-sterilized pecans. The metabolite pattern of the toxic extracts, as determined

by thin-layer chromatography, was the same for the *P. charlesii* isolate whether cultured on pecans or on the shredded wheat medium. Therefore, the latter was arbitrarily selected for mass culture.

A bright-yellow crystalline metabolite (melting point, 96 to 98°C; $[\alpha]_D^{25}$, −78° [C = 0.80 in acetone]) isolated from the ethyl acetate fraction proved to be highly toxic. By thin-layer chromatography, the toxin had an R_f of 0.44 and appeared as a yellow spot in visible light and as a bright-yellow fluorescent spot in long-wave ultraviolet light. The ultraviolet-visible spectra showed λ_{max}^{MeOH} 205, 238, 288(sh), 294, 390, and 802 nm (ϵ_{max} 14,100, 10,000, 22,500, 24,500, 45,000, and 32,000, respectively).

Major absorptions in the infrared spectrum were 3,440 (OH), 2,980 and 1,418 (CH_3), 2,865 cm^{-1} (OCH_3).

High-resolution mass spectrum analysis showed a molecular ion peak at 402.2150 amu (relative abundance, 20.6) (required for $C_{23}H_{30}O_6$, 402.2042). Major fragment ions occurred at nominal masses of 340 (40.6), 302 (31), 285 (7.4), 259 (82.2), 243 (10.9), and 139 (100) amu.

The ^1H nuclear magnetic resonance spectrum gave signals as follows: 1.12 (3H, d, J = 6.2); 1.17 (3H, s); 1.31 (3H, s); 1.91 (3H, d, J = 0.7); 1.96 (3H, s); 3.32 (2H, s, OH); 3.76 (H, q, J = 6.2); 3.89 (3H, s); 5.51 (H, s); 5.95 (H, q, J = 0.7); 6.39 (6H, m); and 6.98 (H, q, J = 15, 8.5) ppm. The ^{13}C nuclear magnetic resonance spectrum was identical within experimental error to that reported recently for citreoviridin (4).

Comparison of these data with data gathered for an authentic sample of citreoviridin and with data presented in the literature (9, 18) showed that the two were identical.

The 50% lethal dose of purified citreoviridin in 1-day-old chickens was 37.5 mg/kg. All mortalities occurred within 18 h after dosing. Animals surviving 5 days appeared to be unaffected by the toxin.

Citreoviridin significantly ($P < 0.01$) inhibited the growth of wheat coleoptiles at concentrations of 10^{-3} and 10^{-4} M (100 and 21.6%, respectively), as compared with controls (Fig. 1). However, coleoptiles were not inhibited at a concentration of 10^{-5} or 10^{-6} M, and citreoviridin was not as active as (±) abscisic acid (2).

Tobacco seedlings were not visibly affected by sprays of citreoviridin, and plants appeared to be normal up to 2 months after treatment. Corn was markedly inhibited at a concentration of 10^{-2} M and slightly inhibited at a concentration of 10^{-3} M (100% and ca. 40%, respectively, as compared with controls) (Fig. 2). The inhibition was still evident 2 months after treatment at a concentration of 10^{-2} M. Thus, preliminary results show that the selective inhibitory properties of citreoviridin may make it an interesting compound for further development as a selective herbicide.

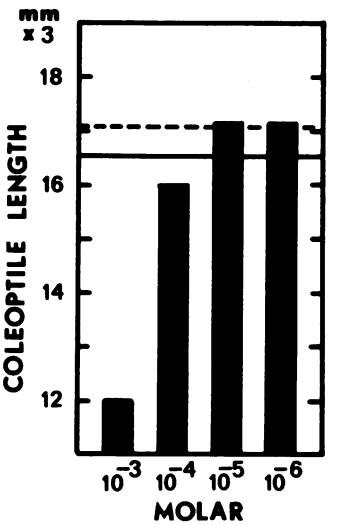

FIG. 1. *Effect of citreoviridin on the growth of wheat coleoptiles.*

Quantitative determination of citreoviridin in 932 g of handpicked pecans showed the toxin to be present at a level of 168.9 mg/kg.

This is the first report of citreoviridin production by *P. charlesii*, although citreoviridin has been reported to be produced by *P. citreo-viride* Biourge (18) (which, with the invalidly named synonymous *Penicillium toxicarium* Miyake, Pitt [11] has assigned to *Penicillium citreonigrum* Dierckx), *Penicillium pulvillorum* Turfitt (9) (= *Penciillium simplicissimum* [Oudem.] Thom, according to Pitt [11]), *P. fellutanum* (8), *Penicillium ochrosalmoneum* Udagawa (17) (the anamorphic state of *Eupenicillium ochrosalmoneum* Scott and Stolk), and *Aspergillus terreus* Thom (4). Citreoviridin has been linked to cases of acute cardiac beriberi in Japan and has also been shown to affect the central nervous system, causing ascending paralysis and respiratory arrest (13, 14, 17, 19).

We were unable to obtain an experimental appraisal of the toxicity of citreoviridin to wild squirrels or birds. The oral 50% lethal doses of citreoviridin for mice and 1-day-old chickens were 29 and 37.5 mg/kg, respectively (18). Assuming a sensitivity of squirrels similar to that of mice, a 0.5-kg squirrel would have to consume approximately 90 g of contaminated pecan fragments to receive the 50% lethal dose. Similarly, a bird weighing approximately 25 g and having the same sensitivity as 1-day-old chickens would

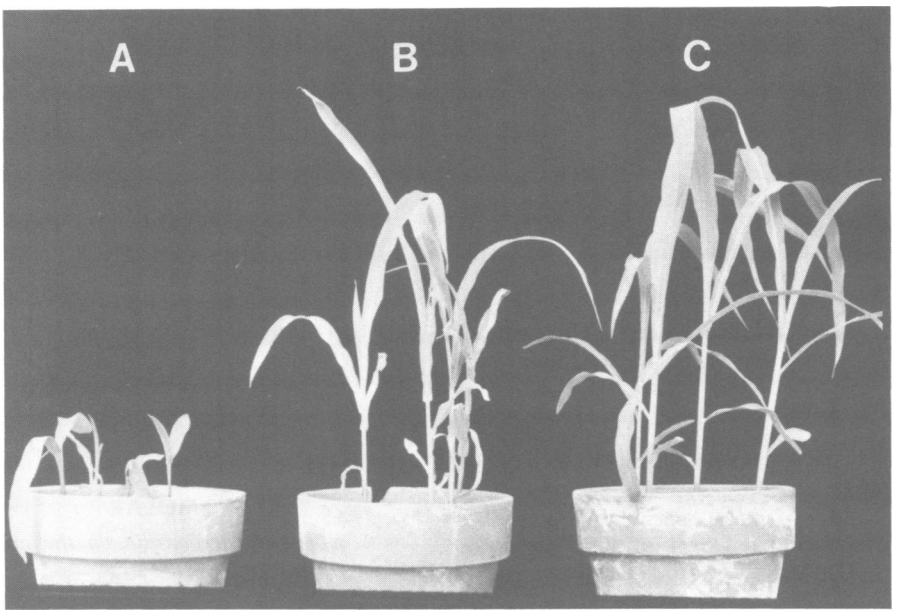

FIG. 2. *Effect of citreoviridin on intact corn plants 14 days after treatment. (A) Citreoviridin at 10^{-2} M; (B) citreoviridin at 10^{-3} M; (C) control.*

have to consume 5.5 g of contaminated pecan fragments to receive the 50% lethal dose.

P. charlesii and *P. verrucosum* var. *cyclopium* grew on the pecan fragments when ambient temperatures during January 1980 were between −1.7 (mean minimum) and 15.8°C (mean maximum). In pure culture on agar, neither species grew at 40°C, but both grew well at 26°C. *P. verrucosum* var. *cyclopium* grew vigorously, whereas *P. charlesii* grew slowly. At 5°C, only *P. verrucosum* var. *cyclopium* grew, although slowly, but with good sporulation. It is likely, therefore, that *P. charlesii* was favored by temperatures close to the ambient maximum, whereas *P. verrucosum* var. *cyclopium* thrived even at ambient temperatures of <5°C. This accords with Hill's (R. A. Hill, Ph.D. thesis, University of Reading, Reading, United Kingdom) observation that various species of *Penicillium* (on barley grain) formed toxins at temperatures closer to the limits for their growth than to the optima.

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