APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1980, p. 882-887
0099-2240/80/04-0882/06$02.00/0
Vol. 39, No. 4

Penitrem A and Roquefortine Production by *Penicillium commune*

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Extracts of *Penicillium commune*, a fungus isolated from cottonseed, showed biological activity in day-old cockerels. Two neurotoxic metabolites were isolated and identified as penitrem A and roquefortine. This is the first report of roquefortine being produced by a fungus other than *Penicillium roqueforti* as well as the first report of penitrem A and roquefortine being produced in the same culture. Production of these toxins on liquid media and cottonseed was determined.

Cottonseed meal is widely used as a protein source in animal feeds and is potentially important as human food. Its use as food has been impeded by the presence of gossypol, which has adverse physiological effects when ingested by monogastric animals, and by the deleterious effects of certain fungal metabolites, namely aflatoxin, which may be present in cottonseed meal (13). In world production of oilseeds, cottonseed is second only to soybeans. If cottonseed meal is to be widely used as an animal feed and have a potential for human usage, information on the mycotoxins produced by fungi on cottonseed becomes important.

Penitrem A and roquefortine are neurotoxic metabolites of several species of *Penicillium* associated with human sources (2, 3, 14, 17). Penitrem A, a known tremorgen, has been found recently in nature on moldy cream cheese (16). Penitrem A has been produced by several species of fungi when grown on corn, wheat, oats, barley, pecans, sausage, and dried beef, but not on peanuts and cottonseed (4, 10). Roquefortine, a paralytic neurotoxin, has been found on blue cheese and has been associated with P-R toxin, produced by *Penicillium roqueforti* (18).

This paper reports on the isolation and production of penitrem A and roquefortine on liquid media and on cottonseed by *Penicillium commune*.

MATERIALS AND METHODS

Organism. P. commune AUA 827, isolated from cottonseed, was used throughout this investigation (9). The culture was maintained at 25°C on agar slants containing 5% sucrose, 0.7% yeast extract, and 0.5% dipotassium phosphate.

Cultivation. *P. commune* was grown in 1-liter flasks in 100 ml of the following media: Czapek-Dox (CD), CD-2% yeast extract (CD-YE), CD-1% corn steep liquor (CD-CSL), 2% yeast extract-4% sucrose (YES-1) (8), 2% yeast extract-15% sucrose (YES-2), and Ushinsky medium (USH) (20). Flasks were stoppered with cotton plugs, and the medium was autoclaved for 20 min at 121°C, cooled, inoculated with 1 ml of a spore suspension from a 7-day-old culture, and incubated at room temperature (22 to 25°C) or a preselected temperature (Table 5). Shake cultures were shaken at 120 rpm in a New Brunswick incubator shaker.

The fungus also was grown on delinted Delta Pine 61 cottonseed, which had been washed, allowed to soak for 2 h in warm tap water, and drained, and 80 g was placed into 500-ml Erlenmeyer flasks. This quantity of thoroughly moistened cottonseed yielded about 50 g (dry weight) of cottonseed per flask. Flasks of cottonseed were stoppered with cotton plugs and autoclaved at 121°C for 20 min, cooled, inoculated with 1 ml of a spore suspension from a 7-day-old culture, and incubated at a preselected temperature.

Relative humidity studies were performed in Blue-M Power-O-Matic (model CFR-7752C) refrigerated humidity cabinets having 10-ft^3 (ca. 0.28-m^3) working chambers. Five cabinets were adjusted to 20°C with relative humidities ranging from 80 to 99 ± 1% at 5% intervals. Approximately 2 kg of cottonseed, which had been washed, soaked, and drained, was placed in each of two perforated trays in the cabinets. The cottonseed was exposed to wet heat at 85°C for 6 h, cooled, and heat treated a second time within 24 h. Cottonseed was inoculated with a suspension of spores by using an atomizer.

Toxin extraction. Chloroform (ca. 100 ml) was added to each flask of moldy cottonseed and to the harvested mycelium from liquid culture, and the cotton plug was replaced. As a safety precaution, flasks were placed on a steam bath under a hood until solvent fumes were evident and for several minutes thereafter to kill spores and mycelium. Contents of each flask were transferred to a Waring blender jar (explosion-proof), covered with 200 ml of chloroform, blended for 1 min, and filtered through 33-cm Eaton-Dikeman grade 615 filter paper. Filtrates were vacuum filtered through anhydrous sodium sulfate, and then concentrated under an air stream on a steam bath under a hood. Liquid medium was extracted twice in a sepa-

ratory flask with an equal volume of chloroform. The solvent was evaporated on a steam bath under a hood.

Toxin purification. The filtered *P. commune* crude extract in chloroform was placed on a 5- by 70-cm glass column packed to a depth of 30 cm with EM Silica Gel G (type 60) in hexane and was sequentially eluted with 1 to 2 liters of each of the following solvents: *n*-hexanes, benzene, diethyl ether, acetone, ethanol, and methanol. A Gilson automatic fraction collector, calibrated to collect 15-ml fractions, was attached to the column. Every tenth tube was bioassayed for toxicity.

Bioassay. Biological activity of the toxins was monitored with day-old White Leghorn cockerels obtained from Chicks of Dixie, Atlanta, Ga. (12). *P. commune* extracts were prepared by evaporating the chloroform on a steam bath under a constant air stream, adding 2 ml of corn oil, and placing in a vacuum oven at 50°C for 12 to 18 h to remove residual solvent. Cockerels were orally dosed via crop intubation with 0.5 ml of extract-corn oil mixture. Cockerels were given food and water ad libitum after dosing. They were observed for 1 to 3 days for changes in gross behavior, but neurotoxic symptoms typically occurred in the first 6 to 24 h.

Toxin characterization. Toxin R_f values were determined in three solvent systems: toluene–ethyl acetate–90% formic acid (TEF) (5:4:1, vol/vol/vol), benzene–acetone (93:7, vol/vol), and benzene–methanol (93:7, vol/vol). A 1-μl sample in methanol was spotted on Whatman MK6F silica gel thin-layer chromatography, 1-inch by 4-inches (ca. 2.54- by 10.16-cm) microslides (Pierce Chemical Co., Rockford, Ill.). The microslides were developed in the appropriate solvent system and air dried. Slides were sprayed with Ehrlich reagent (1% paradimethylaminobenzaldehyde in 95% ethanol), air dried, sprayed with 50% ethanolic sulfuric acid, and heated at 110°C for 10 min or with a hot air blower. Slides were washed with water to remove excess sulfuric acid and then air dried.

An electrothermal melting point apparatus (Electrothermal Engineering Ltd., London, England) was used to determine corrected temperatures for signs of first change, melting, and decomposition.

The ultraviolet and visible absorbancies of methanol-toxin solutions were determined with a Perkin-Elmer model 200 UV-VIS double-beam spectrophotometer with 1-cm cells.

Infrared spectra were obtained with a Perkin-Elmer model 727 B infrared spectrophotometer with NaCl blocks on which the toxin was applied in acetone and dried.

Proton nuclear magnetic resonance (NMR) spectra were obtained on purified compounds in deuterated chloroform containing 5% tetramethylsilane as the internal standard. Data were recorded with an EM 390-MHz NMR spectrometer.

^{13}C NMR analysis was done in deuterated chloroform with a Varian CFT-20 ^{13}C NMR spectrometer.

High-resolution mass spectra were obtained with a Hewlett-Packard model 5985A mass-spectrometer.

Toxin quantitation. An adaption of the methanolysis procedure of Hou et al. (11) was used for quantitation of penitrem A. The toxin in methanol (0.01 ml) was added to a test tube containing 2 ml of methanol and 0.1 ml of concentrated sulfuric acid and heated to 70°C for 20 min; after color development, 2 ml of methanol was added to each tube and cooled, and optical densities were determined at 630 nm with a Perkin-Elmer model 200 UV-VIS spectrophotometer.

Roquefortine was estimated by visual comparisons of known and unknown spots via thin-layer chromatography in benzene–methanol (93:7, vol/vol). A brownish-green spot was observed at R_f 0.31 when the slides were sprayed with Ehrlich reagent and ethanolic sulfuric acid. After a wash with water to remove excess sulfuric acid, a deep blue color developed which was used for the estimation of quantities.

RESULTS

Toxin purification and characterization. Tremorgenic activity in purified extracts was found primarily in fractions 42 and 52, compounds eluted from the column by diethyl ether. Upon evaporation of the solvent at room temperature, this toxin formed long, fine white needles on the sides of the tubes. These crystals were collected and bioassayed, and tremorgenic activity was confirmed in cockerels. Repeated crystallizations from diethyl ether provided pure crystals.

The tremorgenic toxin was identified as penitrem A upon comparison of the following properties with those reported in the literature (2, 6, 7, 23, 24). The compound turned a bright blue on thin-layer chromatographic slides when sprayed with Ehrlich reagent and ethanolic sulfuric acid and heated. The tremorgen had an R_f of 0.4 in benzene–acetone (93:7), 0.5 in benzene–methanol (93:7), and 0.7 in TEF (5:4:1). The tremorgen did not have a precise melting point. The first evidence of melting was at 212°C when a light-brownish color developed, followed by a deep brown at 224°C and dark brown at 232°C. The ultraviolet spectrum showed peaks at 292 and 233 nm in methanol. The major peaks on infrared were 3,290, 2,910, 1,710, 1,625, 1,420, 1,360, 1,225, 960, and 925 cm^{-1}. The proton NMR spectrum was consistent with that reported for penitrem A (8). The mass spectrum indicated a molecular ion at 633 mass units with a base peak at 378.

Paralytic activity was found in fractions 222 and 232, eluents from the column with ethyl acetate–ethanol (1:1). Fractions were dried on a steam bath and taken up in methanol, and light-cream-colored fine needles aggregated as the methanol was evaporated on the steam bath. The methanol solution was reheated till clear and then allowed to cool slowly at room temperature. Crystals that formed were biologically active and were purified by washing with cold methanol, redissolved in warm methanol, and recrystallized.

The paralytic toxin was identified as roquefortine (Fig. 1) on the basis of data reported in the literature (14, 19). The compound was visualized as a greenish-brown spot on thin-layer chromatography plates after spraying and heating as previously described. The paralytic compound had an R_f of 0.1 in benzene–acetone (93:7), 0.3 in benzene–methanol (93:7), and 0.05 in TEF. Authentic standards were chromatographed separately and co-chromatographed with the paralytic toxin; both had the same R_f and did not separate when co-chromatographed. The compound began melting at 202°C and was completely melted at 205°C. The ultraviolet spectrum exhibited peaks at 323, 238, and 210 nm. The major peaks on infrared were 3,200, 2,820, 1,660, 1,595, 1,400, 1,205, 1,060, 1,040, and 910 cm^{-1}. The proton and ^{13}C NMR spectra were consistent with that reported in the literature (14, 19). The mass spectrum indicated a molecular ion at 389 with a base peak at 320.

Bioassay. Crude extract from *P. commune*-contaminated cottonseed caused tremors in day-old cockerels within 30 min after oral dosing. At 2 h postdosing the birds began losing their righting reflex, and within 3 to 5 h a characteristic crooked-neck posture developed where the head was tilted sideways to the body. The birds eventually fell on their side and died within 8 to 12 h after dosing, with their feet flailing, heads and necks extended backwards.

When day-old cockerels were dosed with pure penitrem A, slight tremors began after 1 h, with sustained tremors being evident after 2 h. Sustained tremors in the cockerels had subsided after 24 h, although mild tremors occurred sporadically. At 2 h after dosing with purified roquefortine, animals began to lose their balance, would sit down, and slowly lean to one side. At 5 h after dosing, the animals died with the head and neck extended backwards and the legs and feet extended out from the body.

Effect of environment on growth and toxin production. Penitrem A and roquefortine production by *P. commune* on six liquid media in stationary culture (22 to 25°C) is shown in Table 1. Although YES-1 medium supported higher toxin production than the other liquid media tested, CD-YE and CD-CSL also supported good toxin yields. The pH value of media supporting high toxin production increased rapidly from the initial pH to a pH of about 8 to 9 after 10 days (Table 2). The pH of media supporting low toxin production either rose slowly to a pH of about 8 after 49 days (YES-2, USH) or did not change appreciably from the initial pH (CD). In stationary culture, 97% of the penitrem A and 85% of the roquefortine was formed in the mycelium, whereas little or no toxin production occurred in shake cultures in either the mycelium or the medium (Table 3).

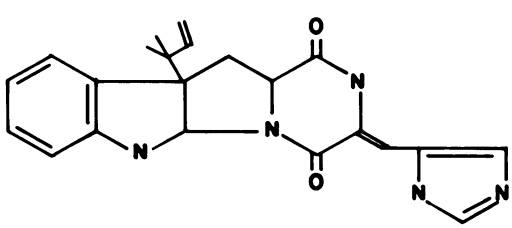

FIG. 1. *Structure of roquefortine.*

TABLE 1. *Effect of different liquid media on toxin production in mycelium of P. commune*

Time (days)	Toxin produced in:					
	CD	CD-YE	CD-CSL	YES-1	YES-2	USH
Penitrem A						
4	0.44[a]	2.00	4.66	3.99	0.66	1.10
7	0.22	3.79	3.24	8.91	0.57	1.33
14	0.01	5.78	3.40	7.82	0.00	0.68
21	0.20	2.60	3.74	8.19	0.21	1.74
49	0.50	5.30	4.22	7.16	1.54	1.82
Roquefortine						
4	0.00	2.03	0.41	0.00	0.00	0.00
7	0.00	6.09	6.09	12.18	0.41	1.22
14	0.00	3.50	5.58	10.15	0.00	0.85
21	0.00	3.04	1.02	10.15	0.10	1.26
49	0.00	3.02	2.03	10.15	0.41	0.67

[a] Data in milligrams of toxin per 100 ml of medium (average of two flasks).

Production of penitrem A and roquefortine in 1-liter flasks containing different media volumes is shown in Table 4. Mycelium of *P. commune* spread more rapidly in flasks containing small amounts of YES-1 medium than in flasks having more medium; consequently, the initial growth rate and initial toxin production were higher at the lower volumes. After 42 days of incubation at room temperature, highest toxin levels (33 mg of penitrem A and 81 mg of roquefortine) were produced with 400 ml of YES-1 medium per 1-liter flask.

The effect of incubation temperature on toxin production of *P. commune* was determined in 1-liter flasks containing 400 ml of YES-1 medium (Table 5). Maximal toxin production (31 mg of penitrem A, 105 mg of roquefortine) occurred after 28 days at 20°C.

The effect of incubation temperature on toxin production by *P. commune* grown on cottonseed is shown in Table 6. Optimum temperature for growth and toxin production was between 15

TABLE 2. *Effect of liquid media on pH with time in cultures of P. commune*

Time (days)	pH in:					
	CD	CD-YE	CD-CSL	YES-1	YES-2	USH
4	4.2	6.1	6.0	3.8	3.9	3.9
7	3.0	7.6	7.3	7.6	3.0	4.0
14	3.9	9.0	8.8	8.9	3.6	4.5
21	3.8	9.1	8.9	9.0	6.5	4.6
49	4.2	8.8	8.8	8.3	8.0	7.7

TABLE 3. *Effect of stationary and shake culture on toxin production by P. commune*

Incubation mode	Toxin production		Toxin in mycelium
	Mycelium	Medium	
Penitrem A			
Stationary	4.73[a]	0.24	97[b]
Shake	0.01	0.01	
Roquefortine			
Stationary	10.15	1.52	85
Shake	0.01	0.01	

[a] Data in milligrams of toxin per 100 ml of YES-1 medium (average of two flasks).
[b] Data in percent.

TABLE 4. *Effect of medium volume per 1-liter flask on toxin production of P. commune grown in stationary culture*

Time (days)	Toxin produced in YES volume/1-liter flask:				
	100 ml	150 ml	200 ml	300 ml	400 ml
Penitrem A					
7	9.12[a]	12.40	3.54	3.28	5.38
14	7.26	11.57	11.94	21.18	16.30
19	8.26	5.48	11.20	16.01	22.39
42	7.45	16.25	17.88	26.74	33.38
Roquefortine					
7	10.15	20.30	0.00	0.00	8.25
14	10.15	30.45	40.60	60.90	60.90
19	40.60	20.30	60.50	81.20	81.20
42	16.24	32.40	48.40	64.80	80.80

[a] Data in milligrams of toxin per flask (average of two flasks).

and 20°C for 35 days when approximately 7 mg of both penitrem A and roquefortine were produced per 50 g of cottonseed. Initial growth and toxin production were good at 25 and 30°C but peaked early. No fungal growth or toxin production was detected at 35°C. Good growth and high toxin production occurred at 5 and 10°C after a long incubation period. Maximum production of 10 mg of penitrem and 20 mg of roquefortine was obtained after 84 days at 10°C.

The effect of relative humidity (RH) on toxin production was determined by growing *P. commune* on cottonseed in open trays incubated in environmental cabinets at 20°C at RH from 80 to 99% (Table 7). After 3 days at 99% RH, the cottonseed substrate was covered with white mycelial growth, and light-green spores were evident after 4 days. Spore color gradually intensified to a dark olive green after 28 days. At 95% RH, cottonseed was covered with the white mycelium after 7 days, and the green spores were evident after 10 days. No visible growth was evident at 90% RH or lower. The optimum RH for toxin production was 99% with production of 11 mg of penitrem A and 4 mg of roquefortine per 50 g of dry cottonseed after 35 days. Moisture content of cottonseed in the environmental cabinets was determined to be 15.1, 22.8, and 32.5%

TABLE 5. *Effect of temperature on toxin production in mycelium of P. commune grown in 400 ml of YES-1 medium in stationary culture*

Time (days)	Toxin produced at:					
	5°C	10°C	15°C	20°C	25°C	30°C
Penitrem A						
14	0.86[a]	2.53	7.91	17.93	10.02	4.70
28	3.34	7.54	23.85	31.29	17.05	2.26
42	5.35	8.44	31.12	20.92	7.88	1.56
Roquefortine						
14	0.53	1.05	10.50	42.00	42.00	10.50
28	2.10	10.50	28.65	105.05	28.65	4.20
42	10.50	38.20	57.30	57.30	19.10	10.50

[a] Data in milligrams of toxin per 400 ml of medium (average of two flasks).

TABLE 6. *Effect of temperature on toxin production by P. commune grown on cottonseed*

Time (days)	Toxin produced at:					
	5°C	10°C	15°C	20°C	25°C	30°C
Penitrem A						
13	0.39[a]	0.80	3.48	2.88	2.83	0.69
28	0.84	4.60	4.60	3.98	2.95	0.38
35	1.48	6.16	6.46	6.95	2.77	0.66
49	2.71	7.36	6.54	5.09	ND[b]	ND
62	6.37	7.82	7.36	2.68	ND	ND
84	8.12	10.48	6.74	ND	1.88	ND
Roquefortine						
13	0.00	0.00	0.00	2.03	6.09	2.03
28	0.00	2.03	5.08	5.08	6.05	2.03
35	0.00	6.05	6.70	3.35	6.05	0.00
49	0.00	11.16	5.58	11.16	0.00	0.00
62	2.03	12.10	12.10	8.12	ND	ND
84	5.73	21.01	9.56	ND	1.91	ND

[a] Data in milligrams of toxin per 50 g of cottonseed (average of two flasks).
[b] Values not determined.

TABLE 7. *Effect of RH on toxin production by P. commune grown on cottonseed*

Time (days)	Toxin produced at RH:				
	80%	85%	90%	95%	99%
Penitrem A					
14	0.00[a]	0.00	0.00	0.22	5.28
28	0.00	0.00	0.00	0.16	11.22
35	0.00	0.00	0.00	0.39	11.03
Roquefortine					
14	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	2.50
35	0.00	0.00	0.00	0.00	3.82

[a] Data in milligrams of toxin per 50 g of cottonseed (average of two flasks).

at 90, 95, and 99% RH, respectively, when determined by the American Oil Chemist's Society method (21).

DISCUSSION

P. commune, isolated from cottonseed, was shown to produce two neurotoxic metabolites, a tremorgenic neurotoxin (penitrem A) and a paralytic neurotoxin (roquefortine), in both liquid culture and on cottonseed. This isolate did not produce ochratoxin or penicillic acid, known mycotoxins of *P. commune* (3, 4).

Ciegler and Pitt (5) tested over 150 different isolates of *Penicillium* spp. in all taxonomic sections to determine whether there was any taxonomic significance to tremorgen production. They observed that only species of *Penicillium* in the section *Asymetrica*, subsection *Fasciculata*, produced penitrem A. It has subsequently been isolated from *P. lanoso-coeruleum* and *P. commune* in the section *Asymetrica*, subsection *Lanata* (4, 22); from *P. canescens* and *P. janthinellum* in the section *Asymetrica*, subsection *Divaricata* (15); and from *P. spinulosum*, which is classified in a different section, *Monoverticillata* (3).

The tremorgenic activity of penitrem A has been demonstrated in many animals (8). Tremorgenic symptoms were obtained in our studies with day-old cockerels dosed with both crude extract and purified toxin from *P. commune*. It has been reported (24) that penitrem A was more effective in 5-day-old than in 3-day-old chickens, and no tremorgenic activity was obtained with day-old chicks. It was suggested that the neural structures necessary for the compound to induce tremors were not developed adequately in day-old chicks to allow tremor induction. Our results refute this claim.

Neurotoxic properties of roquefortine were first reported in mice via intraperitoneal injection (17). Later observations reported no neurotoxic symptoms (1). In our studies, neurological symptoms were observed in day-old cockerels dosed via crop intubation with roquefortine obtained from *P. commune*.

The first natural occurrence of penitrem A toxicosis was reported from moldy cream cheese (16), whereas the natural occurrence of roquefortine in blue cheese has been well documented (17). The co-occurrence of these two neurotoxic compounds has yet to be reported in nature, although we have demonstrated their simultaneous occurrence in culture.

Of the five liquid media tested, YES-1 supported the highest penitrem A and roquefortine levels by *P. commune*. Scott et al. (18, 19) obtained 100 mg of roquefortine per liter with *P. roqueforti* with 2% yeast extract–15% sucrose medium (YES-2), which compares favorably to toxin yields of *P. commune* on YES-1.

Maximum production of penitrem A and roquefortine by *P. commune* on liquid media was obtained with the combination of 400 ml of YES-1 media per 1-liter flask and incubating the stationary cultures at 20°C for 28 days.

Since *P. commune* was isolated from cottonseed, toxin production on this substrate was investigated. Hou et al. (10) reported that no penitrem was produced when four other penitrem-producing fungi were grown on cottonseed. We found that *P. commune* grew on cottonseed and also produced penitrem A and roquefortine. The temperature requirements were the same on cottonseed as on liquid media, and a high relative humidity was needed for moderate toxin production. These data suggest that safe storage could be readily attained by drying the cottonseed to moisture contents below 15%. Although *P. commune* was isolated from cottonseed and produced both penitrem A and roquefortine in our laboratory, it was not determined whether the occurrence of these toxins under field conditions is a significant problem.

ACKNOWLEDGMENTS

We thank R. J. Cole, National Peanut Laboratory, U.S. Department of Agriculture, Dawson, Ga., for authentic penitrem A and roquefortine standards; D. M. Wilson of the University of Georgia Experiment Station, Tifton, Ga., for high resolution mass spectroscopy data; and F. A. Johnson of Chemistry Department, Auburn University, for the high resolution proton and ^{13}C NMR spectra.

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