APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1979, p. 172-173
0099-2240/79/01-0172/02$02.00/0

Vol. 37, No. 1

Tremorgenic Toxins Produced by Soil Fungi

D. S. P. PATTERSON,[1]* B. A. ROBERTS,[1] B. J. SHREEVE,[1] S. M. MACDONALD,[1] AND A. W. HAYES[2]

Central Veterinary Laboratory, Weybridge, Surrey KT15 3NB, England,[1] and Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi 39216[2]

Received for publication 14 September 1978

Penitrem A or an unknown tremorgenic toxin, "X," was produced by 10 of 60 fungal isolates obtained from a pasture involved in an outbreak in cattle and sheep resembling migram and ryegrass staggers. Tremorgenic properties of extracts containing penitrem A or toxin X were confirmed by bioassay.

A syndrome in cattle and sheep resembling migram and ryegrass staggers (1, 2) recently has been reported by Shreeve et al. (8), but during the investigation no tremorgenic toxins were detected in the pasture. However, cattle and sheep normally ingest a certain amount of soil while grazing (5, 6), and, since this disease outbreak occurred during a period of drought when grass was being very closely cropped, the possibility of soil-borne toxins also was considered.

No preformed toxins were detected in seven soil samples (8), but several fungal isolates were found to produce either penitrem A or an unidentified toxin in vitro; this work is described briefly below.

Preliminary cultural examination was carried out as described previously (7), and subcultures of any *Aspergillus* and *Penicillium* species were transferred to slopes of 2% malt extract agar. After incubating at 25°C for 14 days, spores were suspended in 1 ml of phosphate-buffered saline (pH 7.0), and this suspension was used to inoculate 150 ml of liquid medium containing 3% dehydrated mashed potatoes, 2% skimmed milk solids, and 2% sucrose (9).

The cultures, in Roux flasks, were incubated at 25°C for 14 days and thereafter extracted with 200 ml of ethyl acetate, aided by mechanical agitation. Flasks were left overnight at room temperature to allow the solvent to separate, and a portion of each extract (100 ml) was removed, filtered through a bed of anhydrous sodium sulfate, and evaporated to dryness in a rotary evaporator. The syrupy residues then were dissolved in 1 ml of chloroform-methanol (2:1, vol/vol) and tested for the presence of toxins.

Samples of the solutions to be tested and a similar volume (5 μl) of solvent containing approximately 5 μg of authentic penitrem A (a gift from B. J. Wilson, Vanderbilt University, Nashville, Tenn.) were applied to silica gel chromatoplates (MN gel GHR; 250 μm), and thin-layer chromatograms were developed using either (i) diethyl ether-cyclohexane (3:1, vol/vol) or (ii) chloroform-acetone (90:10, vol/vol). An $FeCl_3$ spray reagent (1% in butanol) (9) was used to detect penitrem A as a green spot which gradually appeared at room temperature over a period of 1 to 2 h. The unidentified toxin, "X," appeared as a yellowish-brown spot that fluoresced blue under UV light (365 nm) after spraying with an Ehrlich reagent (1% *p*-dimethylamino benzaldehyde in 96% ethanol) and exposing to HCl vapor for 5 min. R_f values were 0.66 and 0.24 for penitrem A and 0.17 and 0.35 for toxin X in solvents i and ii, respectively.

Table 1 lists 10 isolates found to contain one or the other toxin out of a total of 60 isolates examined. Where the toxin is given as penitrem A, its presence had been confirmed by the characteristic color reaction of a spot on the chromatogram with the appropriate R_f value, by cochromatography with the authentic toxin in four different developing solvent systems (solvents i and ii above; toluene-ethylacetate-90% formic acid; 60:30:10, vol/vol; and toluene-ethylacetate, 1:3, vol/vol), and by UV spectroscopy.

Tremorgenic properties of extracts containing penitrem A or toxin X were confirmed by mouse bioassay. A portion of the crude extract (0.5 ml) was evaporated to dryness, and the residue was redissolved in 0.5 ml of propylene glycol. Up to one-fifth of this solution, when inoculated intraperitoneally or intubated per os, caused tremors (sometimes violent), convulsions, and in a few cases death within a few minutes of administration to albino mice of either sex. One "mouse dose" was equivalent to about 250 μg of penitrem A per kg of body weight. Preparative thin-layer chromatography was used to determine that all toxic activity resided only in chromatographic spots designated penitrem A or toxin X.

When chromatograms were developed on sil-

TABLE 1. *Penicillium isolates producing penitrem A or toxin X in culture*

Isolate no.	Toxin detected by thin-layer chromatography		Mouse bioassay	Fungus identified
	Penitrem A	Toxin X		
170A/28	+	0	+	*P. canescens*[a]
170B/2A	+	0	+	*P. clavigerum*[a]
170C/3	+	0	+	*P. clavigerum*[a]
170C/14	0	+	+	*P. piceum*
170D/2	0	+	+	*P. nigricans*
170D/4	0	+	+	*P. nigricans*
170D/13	0	+	+	*P. piceum*
170G/13	0	+	+	*P. raistrickii*
170G/15	0	+	+	Unidentified *Penicillium* sp.
170G/3	+	0	+[b]	*P. janthinellum*[a]

[a] Probably the first report of these species as producers of penitrem A.

[b] Single observation; all others were repeated at least twice.

ica gel plates containing a fluorescent additive, toxin X appeared as an absorbing spot when viewed in UV light. Purified by preparative thin-layer chromatography, toxin X absorbed light maximally at 223, 274, and 293 nm (methanol). Further studies are currently in progress to determine the identity of this toxin, but since we have recently isolated a similar if not identical compound from *Aspergillus fumigatus* cultures it is distinctly possible that toxin X is fumitremorgen B or a related indole derivative.

A very recent report from New Zealand (3) concerns the isolation of tremorgen-producing penicillia from the soil and feces of animals affected with ryegrass staggers; furthermore, results of our own large-animal experiments (soon to be published) and those of Gallagher et al. (4) suggest that tremorgens produced by soil-borne fungi may be implicated in the pathogenesis of migram, ryegrass staggers, and similar conditions of livestock.

We are grateful to Agnes Onions of the Commonwealth Mycological Institute, Kew, Surrey, England, for identifying the penicillia.

A.W.H. was supported by a N.A.T.O. Senior Fellowship.

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