Production and Some Characteristics of Host-Specific Toxin(s) Produced by Several Isolates of Helminthosporium maydis race T

G. A. Payne and O. C. Yoder

Department of Plant Pathology, Cornell University, Ithaca, NY 14853. Present address of senior author: Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, NE 68583. We gratefully acknowledge the kindness of P. Gregory, Department of Plant Breeding and Biometry, Cornell University, for supplying isolated mitochondria; N. J. M. Erickson and D. Peña for technical assistance; and the U.S. Department of Agriculture (Cooperative State Research Service Grant 216-15-24) and the Rockefeller Foundation (Grants 7422 and 75002) for financial support.

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ABSTRACT

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Isolates of *Helminthosporium maydis* race T from several geographical locations were compared for ability to produce in culture a toxin that is selective against corn containing Texas male-sterile cytoplasm. The isolates did not differ significantly in virulence on corn, growth rate in culture, or rate at which toxin was produced in culture, but small differences were detected in quantity of toxin produced in light versus dark conditions. Toxin preparations were tested for stability at several pH values and were analyzed in several chromatographic systems; there were no apparent differences among preparations from the isolates. There were indications that more than one host-specific toxin could be found in partially purified preparations, but there was no evidence for differences in biological activity or in host-specific activity among different fractions. The instability of these materials in chromatographic systems did not permit reproducible qualitative analyses.

Additional key words: southern corn leaf blight, *Zea mays*.

All isolates of *Helminthosporium maydis* Nisikado and Miyake race T produce host-specific toxin(s) (35) which is thought to be responsible for the high virulence of race T on corn containing Texas male-sterile (T) cytoplasm (22, 34). The toxin (HMT-toxin) is selective for corn containing T-cytoplasm, whereas corn containing nonsterile N-cytoplasm is relatively insensitive to the toxin. However, much of the literature on HMT-toxin is confusing because published reports frequently have contradicted each other, as indicated by the following examples: (i) Some investigators found that HMT-toxin is highly selective for T-cytoplasm corn (6, 7, 33, 37) but others reported either low selectivity or none at all (8, 9, 12, 15, 31). (ii) Sensitivity to HMT-toxin of mitochondria isolated from T-cytoplasm corn (21) has been confirmed many times (3, 4, 10, 19, 23); some investigators indicate that mitochondria in situ also are rapidly affected by toxin (1, 17), but others have found no such evidence and/or have made observations that suggest the plasma membrane as a likely site of toxin action (2, 3, 9, 12, 20, 28). (iii) There is one report (32) that toxin affects respiration of sensitive tissues and another report (3) that it does not. (iv) Attempts to confirm the observation that toxin inhibits membrane-bound ATPase (28) have failed (25). (v) Suggestions of interaction between products of nuclear and cytoplasmic genes for disease reaction (29, 31) have been disputed (22).

The confusion in the HMT-toxin literature may be due to several factors, such as differences in toxin purification procedures or in accuracy and sensitivity of various toxin assays (37). Yet another possibility is that investigators in various laboratories each may have used a different host-specific toxin for physiological studies, because they each used a different isolate of *H. maydis* race T for toxin production. For example, Bhullar and Daly (5) found that light greatly stimulated toxin production by their isolate; our isolate has shown little or no response to light. Furthermore certain isolates of *H. maydis* race T are reported to produce multiple host-specific toxins (12, 13) which differ in biological activity (30). Five host-specific toxins have been reported by Karr et al. (13), based on differential solubilities and chromatographic properties. Toxins I, II, and III were separated from toxin IV by gel filtration. Toxin III was insoluble in acetone whereas toxins I and II were soluble, but were separated from each other by thin-layer chromatography. Toxin V was detected with a chemical assay (14).

The purpose of this study was to investigate the possibility that different isolates of *H. maydis* race T produce different host-specific toxins. The comparative studies were of four types: (i) kinetics of toxin production, (ii) effect of light on toxin production, (iii) effect of different pH values on toxin stability, and (iv) ability of isolates to produce the multiple host-specific toxins described by Karr et al. (13).

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MATERIALS AND METHODS

Fungal isolates and inoculation procedures.—*Helminthosporium maydis* race T isolates MT, D, and F were obtained from G. A. Strobel (Department of Plant Pathology, Montana State University, Bozeman, MT 59715), J. M. Daly (Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, NE 68503), and H. Wheeler (Department of Plant Pathology, University of Kentucky, Lexington, KY 40506), respectively. Isolate NY9 is a field isolate from New York used for routine toxin production in our laboratory. Isolate 111-1-1 was derived from a cross of field isolates from North Carolina (provided by K. J. Leonard, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27607) and New York. For some experiments all five isolates were used, but others were limited to three isolates: NY9, MT, and D. Each isolate was maintained in infected leaf tissue, was freshly reisolated for each experiment, and was grown on potato-dextrose agar in the light for 7 days to produce spores. Spores for inoculation or for addition to culture medium were suspended in sterile water containing 0.01% Triton X-100. Plants (16 days old, 32 plants of each corn genotype) were inoculated by placing 0.2 ml of a spore suspension (25 spores/ml) in each whorl, and held for 9 days in a controlled-environment chamber at 23 C, 80% relative humidity, and 16-hr photoperiod (17.2 klx). The lengths and widths of lesions were measured (35 lesions per isolate), and the area of each lesion was calculated.

Toxin production.—Toxin was produced on the defined medium (D) described earlier (13). Roux bottles, each containing 150 ml of medium D, individually were seeded with 5,000 spores (spores were used to quantify and standardize inoculum) in 1.0 ml of water, and incubated in darkness at 25 ± 1 C for 21 days. In other studies, toxin was produced in 250-ml flasks, each containing 25 ml of modified Fries' medium (33) to which was added 10,000 spores in 1.0 ml of water. Cultures were incubated in a controlled-environment chamber at 25 C either under constant fluorescent light (17.2 klx) or in the dark. Unless otherwise indicated, toxin activity in all filtrates from Fries' medium and medium D, adjusted to pH 4.0, and in all preparations of partially purified toxin, was determined by the dark CO_2 fixation assay (6) with corn inbred W64A in T- and N-cytoplasms. Several dilutions of each sample were used to calculate an ED_{50} value from the linear portion of the dosage-response curve for T-cytoplasm corn (37). In no case was there a detectable effect of toxin on N-cytoplasm corn. Mycelial mats (three/isolate) were dried to a constant weight (60 C for 72 hr) to determine fungal growth.

Stability of toxin at different pH values.—Filtrate from each isolate (grown in the light on Fries' medium for 7 days) was adjusted (with HCl or NaOH) to pH 4.0, 7.0, or 11.0, filter-sterilized, and held aseptically at 25 C. Control filtrates were held at pH 4 and 5 C. Aliquots were removed after 2 hr, 24 hr, and 7 days, adjusted to pH 4.0, bioassayed, and an ED_{50} value was estimated for each. After 2 hr, 24 hr, and 5 days, the pH of each filtrate was checked and readjusted if necessary; pH of filtrates adjusted to pH 11.0 tended to fall with time.

Analysis of toxin produced on defined medium.—The toxin preparations produced by isolates NY9, MT, and D on medium D were each partially purified following exactly the procedures described by Karr et al. (13). The only modification in the procedure was that butanol was removed at 45 C rather than 55 C. The entire purification procedure, from culture filtrate through paper-and thin-layer chromatography and gel filtration (13), was done twice with fresh lots of culture filtrate (1,000 ml/isolate) from each isolate each time. Each residue remaining after the diethyl ether extraction step was dissolved in 1.0 ml of water and applied to a Bio-Gel P-2 [74-38 μm (200- to 400-mesh)] column (1.6 × 80 cm) and eluted with water at a flow rate of 10 ml/hr. Fractions (1.0 ml each) were collected and assayed for toxin activity with the dark CO_2 fixation assay (6) and the leaf-puncture assay (13).

For further analysis, certain toxin-containing fractions (described in Results) were pooled and dried. The residue was resuspended in ethanol (50%, v/v) and applied to Whatman No. 3 chromatography paper. The chromatogram was developed in *n*-propanol-ethyl acetate-water (7:2:1, v/v) (PEAW) and cut into 2-cm wide strips. Each strip was eluted with water and each eluate was bioassayed. Fractions containing toxin were combined and dried under vacuum over P_2O_5. Each residue was mixed with 1.0 ml of acetone and centrifuged to remove an insoluble white precipitate. The precipitate was dissolved in water and the acetone-soluble and acetone-insoluble components were applied to Adsorbosil-5 silica gel thin-layer chromatographic (TLC) plates obtained from Applied Science Laboratories, State College, PA 16801. Manufacture of the Adsorbosil-3 plates used by Karr et al. (13) has been discontinued. Silica gel plates from other sources, including Brinkman (SIL G), Kontes (LQF), and Analab (Anasil GF), gave results comparable to those obtained with Adsorbosil-5. Chromatograms were developed in PEAW and divided into strips 1.0 cm wide. Each strip from chromatograms containing the acetone-soluble or acetone-insoluble components was eluted with 2 ml of acetone or water and bioassayed. Duplicate chromatograms were treated as described by Stahl (26) with one or more of the following spray reagents: iodine-chloroform, anisaldehyde-sulfuric acid, or vanillin-sulfuric acid.

Analysis of toxin produced on Fries' medium.—Culture filtrates from isolates NY9, MT, and D grown on Fries' medium were adjusted to pH 4.0 and extracted five times with an equal volume of chloroform (6, 16). The chloroform phases were pooled and evaporated to dryness. The residue was resuspended in water at 1.0% of the original culture filtrate volume and 1.0 ml was applied to the Bio-Gel P-2 column. Fractions (1.0 ml each) were collected and bioassayed.

Isolation of mitochondria.—Mitochondria were isolated from corn inbred W64A containing T- or N-cytoplasm by the procedure of Gregory et al. (11). Quality of mitochondria was determined by ADP/O ratios, respiratory control ratios (RCR), and respiration rates. With NADH as substrate, the ADP/O and RCR for the second state-3 to state-4 transition were 1.3 and 1.8; with malate as substrate they were 2.0 and 2.4, respectively. The state-4 respiration rates were 23.9 and 53.0 nmoles O_2/mg mitochondrial protein/min for malate and NADH, respectively.

RESULTS

Virulence of fungal isolates.—The five isolates of *H. maydis* were confirmed to be race T by their reactions on a standard set of corn differentials (35). Corn containing T-cytoplasm was more susceptible than corn containing N-cytoplasm and inbred B14A was more susceptible than inbred Mo17. Mean lesion areas (mm^2) of the five isolates on individual corn genotypes were 35 (B14A T), 5 (B14A N), 7 (Mo17 T), and 0.9 (Mo17 N). For each isolate, mean lesion areas (mm^2) of the four corn genotypes were 12 (NY9), 13 (111-1-1), 12 (MT), 12 (D), and 11 (F). All isolates appeared to be equally virulent since differences in sizes of lesions produced by the isolates on a given corn genotype were not significant ($P = 0.05$).

Toxin production.—In cultures exposed to light the five isolates had similar rates of growth and of toxin production, and caused similar changes in pH of the culture medium. Isolates grown in the dark gave curves similar to those for isolate NY 9 (Fig. 1) but produced less toxin. For each isolate, peak toxin activity was correlated with a pH minimum of 4.0 and a peak in fungal dry weight. Except for isolate MT, the rate of toxin accumulation and the quantity of toxin produced were the same for all isolates and were similar to those reported by Bhullar and Daly (5) for light-grown cultures. Isolate MT produced significantly less ($P = 0.05$) toxin both in the light and in the dark than the other isolates (Fig. 2). Incubation in the light rather than in the dark stimulated toxin production by 22 to 31% (Fig. 2). The effect of light was significant ($P = 0.05$) for each isolate, but there were no differences among isolates with respect to light-stimulation. The isolate reported to respond to light with a 7-fold increase in toxin production (5) has since been lost (J. M. Daly, *personal communication*) and was not available for use in this study.

Toxin stability at different pH values.—Toxin preparations from the five isolates of *H. maydis* race T had similar pH stability (Fig. 3). Toxin from each isolate was more stable at pH 4.0 and 7.0 than at pH 11.0. Approximately 95% of the toxin activity was lost at pH 11.0 after 24 hr, whereas 50% of the toxin activity remained at pH 4.0 and 7.0 after 7 days. The experiment was repeated with a new lot of culture filtrate from each isolate. The apparent differences in pH stability among the toxin preparations from the three isolates were not consistent between the two experiments and therefore were not considered significant. There was no loss of toxin activity in filtrate of any isolate maintained indefinitely at pH 4.0 and 4 C.

Analysis of toxin produced on defined medium.—When produced by the procedure reported by Karr et al. (13), culture filtrates contained only 15% as much toxin as those produced on Fries' medium, which confirmed previous observations (5, 36). These low toxin titers severely restricted the amount of material available for purification and analytical procedures. In addition, over 90% of the initial toxin activity was lost in the purification procedure itself (as measured by the dark CO_2 fixation assay), even when preparations were dried at 45 C. Drying at 55 C, as prescribed (13), resulted in loss of over 99% of the toxin activity.

Bio-Gel chromatography.—Toxin activity remaining after solvent extraction was analyzed by the series of

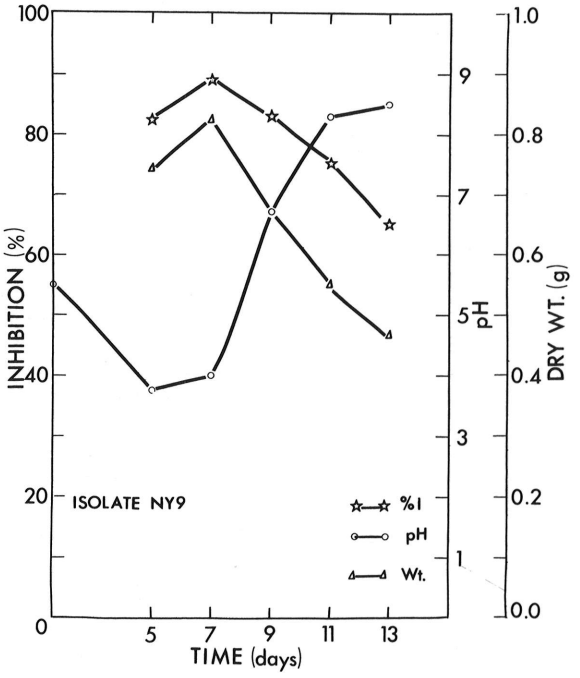

Fig. 1. Performance of *Helminthosporium maydis* race T isolate NY9 grown in the light in Fries' medium. Percent inhibition (% I) indicates the effect of culture filtrate (diluted 1:1,000) on dark CO_2-fixation. Dry weight (wt) represents the total weight of three mycelial mats. The pH, wt, and % I curves shown here for isolate NY9 are not significantly different from those for isolates 111-1-1, MT, D, and F grown on Fries' medium.

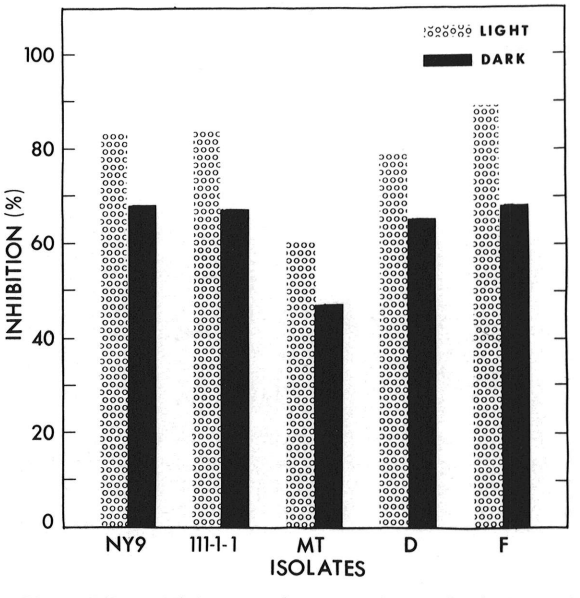

Fig. 2. Effect of light on toxin production by five isolates of *Helminthosporium maydis* race T. Toxin activity in culture filtrate (diluted 1:1,000) was determined with the dark CO_2-fixation assay and is expressed as percent inhibition. Filtrates were harvested after 7 days of fungal growth in Fries' medium.

chromatographic techniques described by Karr et al. (13). On Bio-Gel P-2 (Fig. 4), toxin activity eluted as a broad band, apparently composed of overlapping peaks. The first two peaks (fractions 67 to 79, peak 1, and 80 to 88, peak 2) were resolved in a single preparation from each of the three isolates in each of two separate sets of column runs and in a third run with only the preparation from isolate NY9. The smaller peaks (peaks 3 and 4) appeared only in one column run (Fig. 4). In the other experiments, this area of the profile was occupied by the trailing edge of peak 2. Peak 1 and peak 2 were concentrated to 1.0 ml each, and rechromatographed on Bio-Gel P-2. Each peak eluted at its original V_e/V_o value (Fig. 4) suggesting that peaks 1 and 2 are distinct from each other. The entire purification procedure, including the Bio-Gel P-2 column, was repeated with new lots of culture filtrate from isolates NY9, MT, and D. Again, peaks 1 and 2 were apparent in preparations from all three isolates, but the minor peaks (peaks 3 and 4) were not.

Fractions (nondiluted) from each of several column runs were assayed separately by the leaf puncture method (13) in an attempt to identify peaks X and Y, reported previously (13). Activity was found in fractions 65 through 100, which corresponds approximately with the migration of peaks X and Y (13), if the fraction numbers are adjusted by 15 to correct for differences in position of void volume. Glucose eluted closer to toxin activity in the previous work (13) than in our case (Fig. 4), although this difference may be due, in part, to the narrower band of toxin activity that we observed. The overlapping peaks (peaks 1 and 2) resolved by the dark CO_2-fixation assay (Fig. 4) were not detected with the leaf-puncture assay, probably because of the nonquantitative nature of the leaf puncture assay (6, 37).

Paper- and thin-layer chromatography.—We attempted to identify toxins I, II, and III, which are reported to be found in peak X (13). For preparations from isolates NY9, MT, and D, fractions from the Bio-Gel P-2 column containing overlapping peaks 1 and 2 were pooled and dried. The residue from each preparation was dissolved in 50% ethanol (200 μliters), separated on chromatography paper in PEAW, and the paper was cut into strips 2-cm wide. Each strip was leached in water and the water fractions were bioassayed. Toxin from each of the three isolates migrated as a broad band similar to that described by Karr et al. (13). For a typical experiment, R_f values were 0.56 to 0.94 (isolate NY9), 0.68 to 0.97 (isolate MT), and 0.70 to 0.98 (isolate D). Differences among isolates were not significant.

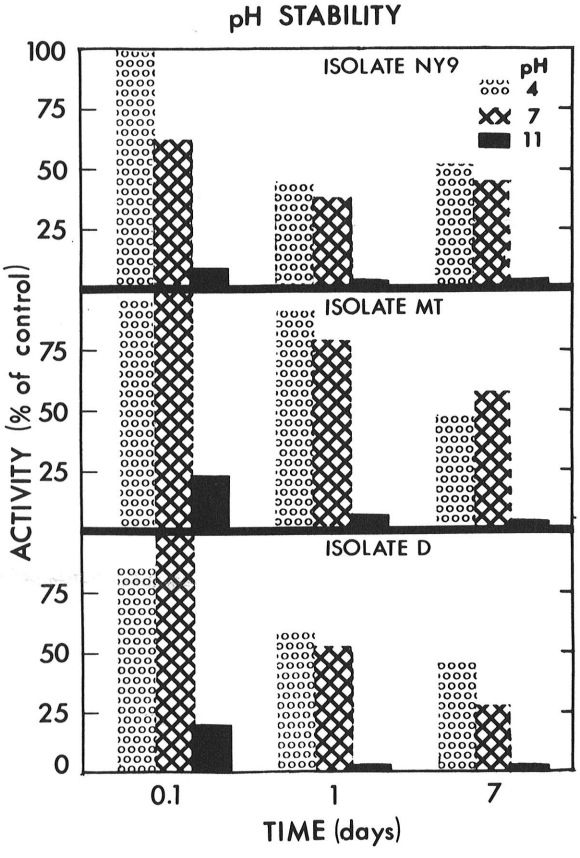

Fig. 3. Stability at different pH values of toxin in culture filtrates from *Helminthosporium maydis* race T isolates NY9, MT, and D. Filtrates were held aseptically at 25 C and sampled at 0.1, 1, and 7 days. Several dilutions were made from each sample and were tested with the dark CO_2-fixation assay. Dilutions which fell on the linear portion of the dosage-response curve were used to calculate the dilution required for 50% inhibition of CO_2-fixation. That dilution was compared to the dilution of a control toxin solution (maintained at pH 4.0 at 4 C) which caused 50% inhibition, and activity was expressed as percent of control. Filtrates from isolates 111-1-1 and F gave results similar to those shown here.

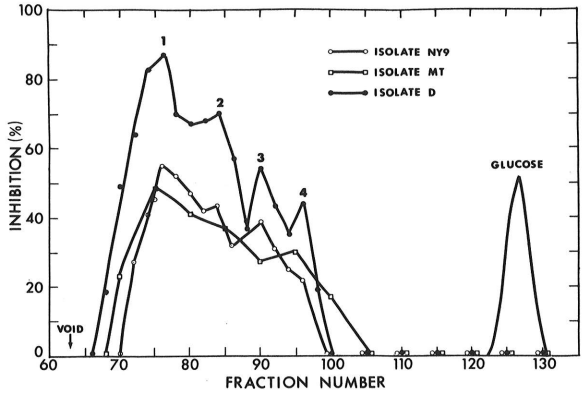

Fig. 4. Fractionation on Bio-Gel P-2 of partially purified toxin preparations from *Helminthosporium maydis* race T isolates NY9, MT, and D grown on medium D. After purification through the diethyl ether extraction step (13), the preparation from each isolate was dissolved in 1.0 ml of water and applied to the column; ED_{50} values of the preparations (tested with the dark CO_2-fixation assay) from isolates NY9, MT, and D were 1:5,000, 1:500, and 1:10,000, respectively, before passage through the column. Each fraction (1.0 ml) was tested for toxin activity (percent inhibition) with dark CO_2-fixation assay after dilution of 1:30 (isolate NY9), 1:4 (isolate MT), or 1:60 (isolate D). No toxin activity was detected in the void volume or in fractions 105 to 200 (each fraction was diluted 1:6 for assay) for any of the isolates.

To test for toxins I, II, and III (13), the toxin preparations recovered from paper chromatograms were treated with acetone and analyzed by thin-layer chromatography (TLC), following the procedures described by Karr et al. (13). Toxin-containing fractions from each isolate were pooled and dried in vacuo over P_2O_5. Each residue was resuspended in 1.0 ml of acetone and centrifuged. The supernatant fluids (acetone-soluble fractions) each had a faint yellow color, whereas the residues (acetone-insoluble fractions) were recovered as white powders. The precipitates were dissolved in water, and both acetone-soluble and acetone-insoluble fractions from each isolate were separated by TLC in PEAW. Bands of gel 0.5 cm wide were removed, eluted in water (acetone-insoluble material) or acetone (acetone-soluble material), and bioassayed. For both acetone-soluble and acetone-insoluble preparations, values of R_f and $R_{cholesterol}$ (migration relative to that of cholesterol), respectively, were 0.65 to 0.75 and 0.85 to 0.98 (isolate NY9), 0.65 to 0.75 and 0.85 to 0.98 (isolate MT), and 0.60 to 0.75 and 0.78 to 0.98 (isolate D). Approximately 98% of the host-specific toxin activity was recovered from the TLC plate to which the acetone-soluble fraction was applied. The remaining activity was detected by bioassay in the acetone-insoluble fraction.

We considered the possibility that peak 3 from the Bio-Gel column (Fig. 4) was different from peaks 1 and 2 and comparable to toxin III of Karr et al. (13). Fractions 89 to 93 from isolate D (Fig. 4) were pooled, dried, resuspended in 50% ethanol, and separated by TLC with PEAW. The gel was divided into bands (1.0 cm wide), each band was leached in 50% ethanol, and the leachate was dried, resuspended in water, and bioassayed. The toxin migrated as a diffuse band from R_f 0.53 to 0.80; considering variation among tests, this migration rate was not different from that of toxin in peaks 1 and 2.

Toxins I and II (13) are reported to have $R_{cholesterol}$ values of 0.8 and 0.7 on TLC plates developed with PEAW. The broad band of toxin activity located on TLC plates at about $R_{cholesterol}$ 0.80 to 0.98 in our tests may correspond to toxins I and II. In one experiment, this band of activity from a single plate was rechromatographed (TLC) three successive times in PEAW and fractions were bioassayed; in each case the broad band was not resolved. The resolving power of the TLC system was tested, however, and would separate the closely related compounds cholesterol and coprostanol. Failure to resolve the diffuse band of toxin activity was not due to overloading; the single diffuse band appeared at approximately the same location regardless of toxin

TABLE 1. Thin-layer chromatography of *Helminthosporium maydis* race T-toxin: detection of host-specific toxin activity and of spray-reactive zones[a]

Migration (cm)	Inhibition[b] (%)	Spray reaction[c]
1	3	
2	0	
3	1	
4	3	
5	8	
6	0	
7	3	
7.5	0	+
8.0	6	
8.5	4	
9.0	17	
9.5	31	+
10.0	47	
10.5	54	
11.0	48	
11.5	21	+
12.0	7	
13.0	0	
14.0	3	
15.0	8	

[a]Toxin, produced on medium D and purified through the solvent extraction, Bio-Gel and paper chromatography, and acetone-treatment steps (13), was applied to duplicate TLC plates which were then developed to a height of 15 cm with PEAW. Cholesterol, included as a standard, migrated 11.7 cm. One plate was sprayed with iodine (0.5 g) in chloroform (100 ml). Gel on the other plate was removed in bands which were then leached in acetone. The acetone was removed, each residue was suspended in water, diluted 1:40, and tested with the dark CO_2-fixation assay.

[b]Inhibition of dark CO_2-fixation by each fraction was determined by comparison with a control. Inhibition <10% was not considered significant.

[c]Symbol + = visible color; blank = no visible color.

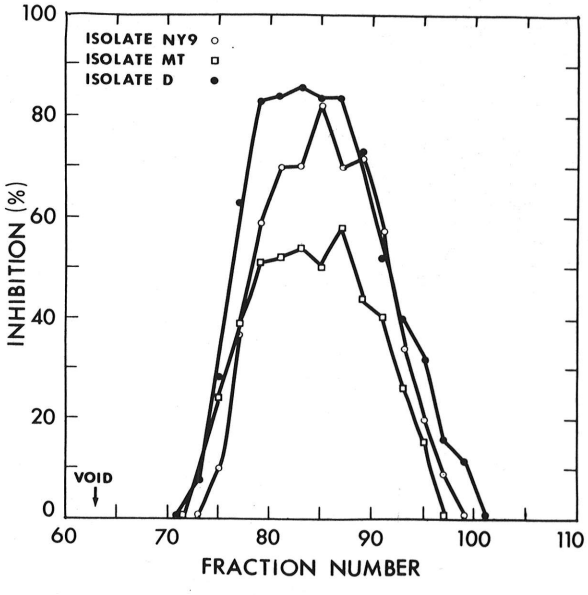

Fig. 5. Fractionation on Bio-Gel P-2 of partially purified toxin preparations from *Helminthosporium maydis* race T isolates NY9, MT, and D grown on Fries' medium. Toxin was extracted from culture filtrates with chloroform and applied to the column in water (1.0 ml). Volumes of the preparations were adjusted such that each had an ED_{50} in the dark CO_2-fixation assay at a 1:1,000 dilution prior to passage through the column. Fractions (1.0 ml) were collected, diluted 1:120 (for isolates NY9 and D) or 1:240 (for isolate MT), and tested for toxin activity (% inhibition) with the dark CO_2-fixation assay. No toxin activity was detected in the void volume or in fractions 102 to 160 (each fraction was diluted 1:120 for assay) for any of the isolates.

quantity, even when applied at the lower limit of detection of the bioassay. When toxin preparations chromatographed on TLC plates were sprayed with iodine in chloroform (or with anisaldehyde-sulfuric acid or vanillin-sulfuric acid), multiple spots were observed (Table 1), but these spots did not correspond to the diffuse band of toxin activity.

Analysis of toxin produced on Fries' medium.—*Bio-Gel chromatography.*—Isolates NY9, MT, and D were grown on Fries' medium and each of the culture filtrates was extracted with chloroform. The chloroform fractions were pooled and dried, the residue was resuspended in water, and applied to a Bio-Gel P-2 column. The elution profiles for toxin preparations from the three isolates were the same (Fig. 5). The profiles were similar to those for toxin produced by the same isolates on medium D (Fig. 4), except that multiple peaks were not observed.

Thin-layer chromatography.—Partially purified toxin produced on Fries' medium was analyzed by TLC. With PEAW, toxin activity migrated as a diffuse band from R_f 0.63 to 0.77. Considering variation in R_f values among experiments, this migration is about the same as that of toxin preparations from medium D. In an attempt to resolve toxin activity into more than one component, three chromatographic supports (thin-layer silica gel, thin-layer cellulose, and paper) were tested in combination with 16 solvent systems. In all these combinations except one, toxin migrated as a single broad band. In one system, Adsorbosil-5 TLC plates developed with butanol-acetic acid-water (6:3:1,v/v), two diffuse bands of host-specific activity sometimes were resolved (Table 2). When these bands were recovered and rechromatographed in the same system, activity in each band was resolved into multiple distinct areas. However, in this and many additional tests, the appearance of any particular "toxin" was not predictable and the rates of migration in the TLC system were highly variable from one run to the next (Table 2).

Effect of toxin on mitochondria.—Throughout this study biological activity was identified with the dark CO_2-fixation assay. If multiple toxins exist, it is important to know if they all have the same biological activity, especially since there is a report that toxin III of Karr et al. (13) does not affect mitochondria (30). To evaluate our fractions, mitochondria were isolated from T- and N-cytoplasm corn and tested against toxin from the four peaks of activity in the Bio-Gel profile of isolate D (Fig. 4). Fractions were mixed with mitochondria (after their second state-3 to state-4 transition) at a concentration about three times the ED_{50} in the dark CO_2-fixation assay. None of the fractions affected mitochondria from N-cytoplasm corn, whereas all of the fractions caused effects on mitochondria from T-cytoplasm corn. Malate oxidation was inhibited (mean inhibition was 65%), NADH oxidation was stimulated (mean stimulation was 230%), and ADP/O and RCR for both substrates were reduced; each of these effects is characteristic of HMT-toxin (4, 21, 23).

DISCUSSION

Our results do not support the idea that different *H. maydis* race T isolates produce different toxins with

TABLE 2. Fractionation of *Helminthosporium maydis* race T-toxin by repeated chromatography on thin-layer plates developed with butanol:acetic acid: water (6:3:1,v/v)[a]

	R_f of toxin activity					
	First TLC		Second TLC		Third TLC	
Experiment	Band I	Band II	Band I	Band II	Band I	Band II
1[b]	0.20 to 0.33	0.60 to 0.80	0.13 0.33 0.47 0.60	0.20 0.40 0.67		
2[c]					0.07 0.40 0.67 0.80	0.13 0.33 to 0.80
3[c]					0.13 0.40 0.60 0.80	0.93

[a]Toxin preparation (37) was applied to TLC plates, which then were developed with solvent to a height of 15 cm. Gel was removed (1.0-cm sections) from plates and leached in acetone. Each fraction was dried, the residue dissolved in water, and tested with the dark CO_2-fixation assay. All fractions reported here to have toxin activity caused >20% inhibition of CO_2 fixation; inhibition <10% was considered insignificant.

[b]After the first TLC run, toxin activity was found in two diffuse bands separated by a large area with no detectable toxin activity. Toxin in each band was pooled, dried, dissolved in acetone and reapplied to a separate TLC plate (second TLC) which was developed and tested for toxin. The plates to which bands I and II, respectively, were applied showed four and three distinct areas of toxin activity separated by areas with no detectable activity.

[c]Procedures were similar to those described for Experiment 1, except that fractions were not bioassayed after the first and second TLC runs. Instead, after each of these runs gel from 0 to 6 cm (band I) and 7 to 12 cm (band II) was pooled separately, leached in acetone, and material from each band was reapplied to a separate TLC plate. After the third TLC, gel was removed and tested for toxin by bioassay.

similar host-specificity. In culture, the isolates that we compared were similar with respect to rate of toxin accumulation, quantity of toxin accumulated, light-stimulation of toxin accumulation, and relative ability to produce toxin on different culture media. Toxin preparations from the isolates had similar stabilities at various pH values and had similar but unpredictable migration patterns in a variety of chromatographic systems. Contradictory evidence in the literature on HMT-toxin may result from factors other than qualitative variation in toxin production by isolates of *H. maydis* race T.

Although our data support previous suggestions (12, 13) that multiple host-specific toxins may exist in preparations from *H. maydis* race T, our results differ in some respects from those of Karr et al. (13). They reported two widely separated peaks by column chromatography, an acetone-insoluble toxin, apparently predictable behavior of multiple toxins in thin-layer chromatographic systems, and recovery of 90% of original toxin after partial purification. With similar analytical procedures (but a more sensitive toxin bioassay) we found two closely overlapping peaks by column chromatography, questionable evidence for an acetone-insoluble toxin, unpredictable behavior of multiple toxins in thin-layer chromatographic systems, and loss of about 90% of original toxin activity during purification. Other investigators (e.g., 16) reported additional differences in results of toxin isolation procedures.

Some of the differences in results of TLC analyses may have resulted from our use of Adsorbosil-5 instead of Adsorbosil-3, which is no longer available. We have been informed (A. L. Karr, *personal communication*) that Adsorbosil-3 is important to obtain the results previously reported (13). Another possible explanation for differences in results involves differences in methods employed to detect toxin. We used the dark CO_2-fixation assay for analytical work because it is quantitative, consistent among experiments, and highly sensitive to toxin (6, 37). The leaf-puncture bioassay used by Karr et al. (13) is not quantitative (6, 37), is variable among experiments, and is at least 100 times less sensitive to toxin than the dark CO_2-fixation assay. Spray reagents and other chemical assays (14) do not reliably detect toxin activity (36). Differences in recovery of toxin in the purification procedure also can be explained on the basis of different methods of detection; we quantified toxin activity with the dark CO_2-fixation assay whereas Karr et al. (13) based their estimate on recovered radioactivity, not biological activity.

In the previous study (13), the leaf-puncture bioassay was used to resolve peak X (toxins I, II, and III) from peak Y (toxin IV). Since the bioassay is nonquantitative, and since there apparently was toxin activity between peaks X and Y (13), it is possible that the area occupied by peaks X and Y represents a broad band of activity corresponding approximately to the toxin-containing fractions found in our study (Fig. 4); toxin IV may not be a distinct entity. Similarly, our preparations did not appear to contain detectable amounts of material analogous to toxin III reported earlier (13). The acetone-insoluble powder contained only a trace of toxin activity, which may have been due to cross contamination; this activity migrated in TLC with the acetone-soluble material.

There are reports that toxin preparations from several other host-specific toxin-producing fungi contain multiple host-specific entities (18, 24, 27). However, in none of these cases (including HMT-toxin) is the biological significance (if any) of multiple toxins apparent. It may be an artifact of isolation and purification procedures; in the case of HMT-toxin the chemical environment may determine the nature of the multiple components (J. M. Daly, *personal communication*). If the fungus actually produces multiple host-specific chemicals, they all may have the same biological effects and thus the same role in disease. Fractions from peaks of HMT-toxin activity on the Bio-Gel column (Fig. 4) all caused the same characteristic effects on both dark CO_2-fixation and mitochondria. Until the chemical nature of the toxin(s) is known and/or there is convincing evidence that disease development depends on more than one host-specific chemical, there is no basis for making biological distinctions among various toxic fractions.

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