T-Toxin Production by Near-Isogenic Isolates of *Cochliobolus heterostrophus* Races T and O

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ABSTRACT

Tegtmeier, K. J., Daly, J. M., and Yoder, O. C. 1982. T-toxin production by near-isogenic isolates of *Cochliobolus heterostrophus* races T and O. Phytopathology 72:1492-1495.

Mycelia and culture filtrates from four near-isogenic isolates of *Cochliobolus heterostrophus* (*Helminthosporium maydis*), segregating monogenically for race differences, were extracted for host-specific toxin known to be produced by *C. heterostrophus* race T. Extracts from the race T isolates produced large amounts of an acetone- and chloroform-insoluble white precipitate that was chemically and biologically identical to previous preparations of T-toxin. Although a slight precipitate sometimes formed in extracts from cultures of the race O isolates, this precipitate was nontoxic. Unprecipitated material remaining in extracts of race T cultures also exhibited specific toxicity, but similar fractions of race O cultures did not. The mycelium of race T isolates accounted for 95% of the toxin extracted, which conservatively can be estimated to amount to 2% of mycelial dry weight. Thus, the single gene shown to determine virulence of these isolates to corn with Texas male-sterile cytoplasm also appears to control the abundant production of a chemically defined product that is essential for high virulence, but not for fungal growth or survival.

Additional key words: *Bipolaris maydis, Drechslera maydis*, HmT-toxin.

In 1976, Kono and Daly (7) first reported the purification of the host-specific toxin (T-toxin) from *Cochliobolus heterostrophus* Drechsler race T [anamorphs: *Bipolaris maydis* (Nisik.) Shoemaker = *Helminthosporium maydis* Nisik. = *Drechslera maydis* (Nisik.) Subram. and Jain]. T-toxin is specific for corn (*Zea mays*) having Texas male-sterile cytoplasm. The toxin has been chemically characterized as a family of long-chain (C_{35}–C_{45}) polyketols (8–10). The four major components have been structurally characterized (8–10). These compounds are all host-specific and toxic at 10^{-8} to 10^{-9} M (8,13).

The available biological and chemical evidence makes the presence of an unrecognized host-specific toxic contaminant in the T-toxin preparations unlikely. However, as with all natural products, this possibility must be considered. Two approaches have been used to eliminate the concern over contamination: laboratory synthesis of toxin analogs and demonstration of their host-specific activity (16,17), and demonstration of the validity of the isolation procedure for T-toxin. This report presents data in support of the second approach.

C. heterostrophus race T is sexually compatible with race O of that species; race O never has been reported to produce T-toxin. From crosses between the two races, it has been determined that race differences and the presence of T-toxin in culture filtrate are absolutely linked and can segregate from other traits monogenically (12,20). However, other work (1, and *unpublished*) indicated that mycelium of race T isolates contained appreciable T-toxin, and it is not known whether the lack of activity in culture filtrate is indicative of the presence or absence of T-toxin in the mycelium. Failure to detect activity in culture filtrate could arise from an inability to secrete toxin. In addition, the chemical nature of the host-specific toxin was not established in the earlier work (12,20). *Phyllosticta maydis* also produces a toxin of unknown composition with the same host-specificity (4,18). This observation raises the possibility that the same specificity may occur with chemically distinct toxins. In fact, the insecticide methomyl, while structurally quite different from T-toxin, has the same specificity to corn having Texas male-sterile cytoplasm, although at much higher concentrations (10^{-3} M) (6). In this study, cultures (filtrates plus mycelium) of near-isogenic isolates of *C. heterostrophus* races T and O, segregating monogenically for race differences, were extracted by using the procedure of Kono and Daly (8) for purifying toxin. Extracts were then examined for the presence of T-toxin with properties similar to those reported by Kono and Daly (8).

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MATERIALS AND METHODS

Sources and culture of isolates. Four near-isogenic isolates were produced by backcrossing race O for six generations to a recurrent race T parent. Progeny from the sixth backcross were scored for segregation at three unlinked loci, each with two alleles: race (*TOX*1/*tox*1), mating type (*MAT*A/*MAT*a), and albino (*ALB*1/*alb*1) (12,20). Isolates C4 (ascospore number 141-2-1) and C6 (ascospore number 141-2-4) were race T and had the genotypes *MAT*a, *ALB*1 (pigmented), *TOX*1 (produced toxin) and *MAT*A, *alb*1 (albino), *TOX*1, respectively. The two race O isolates were C7 (ascospore number 141-4-2; genotype *MAT*A, *alb*1, *tox*1 [produced no toxin]) and C8 (ascospore number 141-4-5; genotype *MAT*a, *ALB*1, *tox*1).

Isolates were maintained and conidial suspensions were made up as previously described (2). Low-form culture flasks (2.5-L) containing 400 ml of Fries' medium (15) supplemented with 0.1% yeast extract were inoculated with conidial suspensions and incubated at 28 ± 1 C and 1,150 lux. Time of harvest varied from 15 to 28 days after inoculation. Four flasks of the race T cultures and five to 12 flasks of race O cultures were used in each experiment, since culture filtrates of race O have no detectable T-toxin activity. Extractions were done twice with the race T cultures, four times with the race O cultures.

Extraction of T-toxin. All glassware used for growing fungal cultures and isolation of toxin was washed normally, then rinsed with 10% KOH or hot methanol to remove traces of T-toxin adhering to the glassware (14).

The isolation procedure of Kono and Daly (8) was used with slight modifications. Both mycelia and culture filtrates were extracted (8) because Bhullar (1) had noted earlier that the

mycelium contained considerable toxin. Mycelia were separated from culture filtrates by filtering through Miracloth (Calbiochem-Behring Corp., LaJolla, CA 92037). Culture filtrates were treated with 3% Norit A (w/v); after 1 hr, the Norit was separated from the solution by filtration and the filtrates were discarded. Mycelia were extracted two to five times by homogenization in 500 ml of 70% acetone and heated to boiling. The acetone extracts were pooled and treated for 1 hr with the same Norit A used in the extraction of the culture filtrates. The Norit-treated extracts were filtered through Whatman #1 filter paper, the Norit was A rinsed with 100% acetone, and the toxin was eluted from it by extracting two to three times with 1,000 ml of 5% methyl alcohol in chloroform; the eluates were pooled and dried in vacuo at 45 C with a rotary evaporator. The resultant oily residue was dissolved in 4–40 ml of either warm chloroform or acetone. Upon cooling, a white to slightly yellow precipitate formed in all extracts from race T cultures. In one experiment, the precipitate was collected by filtration. In all other experiments, the total activity was determined by warming the extracts from race T and race O cultures under hot tap water to dissolve any precipitate. Aliquots were withdrawn, the solvent was removed under a stream of N_2, and the residues were dissolved in dimethyl sulfoxide (DMSO). In one experiment, culture filtrates and mycelia were extracted separately for cultures of all four isolates.

Thin-layer chromatography was performed by using Merck EM 60 plates and chloroform-methanol (94:6) as solvent (8) on precipitates from isolates C4 and C6 and on a toxin preparation made previously by J. M. Daly (T-toxin 5/5/79).

Bioassay by inhibition of dark CO_2 fixation. Extracts were tested for host-specific toxicity by using a bioassay for inhibition of dark CO_2 fixation in thin corn leaf slices. Sensitive (Tms-cytoplasm) and insensitive (N-cytoplasm) plants of inbred W64A corn were grown as described previously (3). Fifteen thin leaf slices from the fourth leaf were placed in 7-ml scintillation vials containing 475 μl of buffer solution, pretreated with extracts for 60 min in the light, and allowed to fix $^{14}CO_2$ supplied as 25 μl of $NaHCO_3$ for 15 min in the dark (5,17). Extracts in 1–4 μl of DMSO were added to vials and compared to a control containing DMSO alone. Culture filtrates were diluted in distilled water, added to vials in 1–4 μl volumes, and compared to control vials supplemented with an equivalent volume of water. Controls were run in triplicate, test solutions in duplicate. Standard deviations were no more than 10% of the rates of fixation in each assay. Data presented are the means of a minimum of two assays.

RESULTS

After removal of mycelium only, culture filtrates from the four near-isogenic isolates were bioassayed for T-toxin activity on sensitive (Tms-cytoplasm) and insensitive (N-cytoplasm) corn leaf slices (Table 1). Culture filtrates from isolates C4 and C6 caused specific inhibition of dark CO_2 fixation at 1,000-fold or greater dilutions, thereby verifying their phenotype as race T. No specific inhibition of Tms-cytoplasm corn was observed with the culture filtrates of isolates C7 and C8 at 125-fold dilutions, indicating that these isolates are race O. Tms- and N-cytoplasm plants were inoculated with all four isolates; small lesions were produced by isolates C7 and C8 on Tms- and N-cytoplasm corn and by isolates C4 and C6 on N-cytoplasm corn, but isolates C4 and C6 showed the enhanced virulence typical of race T on Tms-cytoplasm corn.

Extracts from cultures (filtrate and mycelium) of both race T isolates produced large amounts of an acetone-insoluble white precipitate that was chromatographically identical in the number and R_f values of components to a standard toxin precipitate prepared earlier (T-toxin 5/5/79). In addition, the infrared spectra of toxins prepared from the isogenic lines was indistinguishable from that of 5/5/79 toxin (Fig. 1). Precipitates from both cultures also were bioassayed for activity on Tms-cytoplasm corn over a range of concentrations; they were not significantly different from one another, nor from the standard toxin preparation (Fig. 2). At 10 ng/ml, all of the preparations caused approximately 30% inhibition of dark CO_2 fixation on Tms-cytoplasm corn, and inhibition increased logarithmically up to approximately 100 ng/ml. At a concentration of 1,000 ng/ml none of the preparations caused significant inhibition on N-cytoplasm corn. Not all the toxin had precipitated from acetone, however, since the acetone-soluble material from race T isolates inhibited dark CO_2 fixation but at 25-fold greater concentrations (Fig. 2). This material did not inhibit dark CO_2 fixation in N-cytoplasm corn at 10,000 ng/ml. Based on the relative activities of the precipitates and soluble fractions, approximately 75% of the total host-specific toxin was collected in a precipitate of known composition.

Purified toxin precipitates from cultures of isolates C4 and C6 resulted in yields of 15 and 21 mg/g of mycelial dry weight, respectively, or 1.5–2.1% of the fungal dry weight. Over 95% of this appeared to be derived from the mycelium, as was determined in the second extraction of cultures of the two race T isolates in which the mycelium and culture filtrate were extracted separately. The precipitates were dissolved in chloroform and the total extracts were bioassayed. The mycelial extracts from the race T cultures had approximately threefold more solids than those from the culture filtrates (Table 2) and were 10-fold more active on a dry weight basis (Table 3). Extracts from the mycelia of the two race O isolates had fewer solids than those from the race T isolates (Table 2) and neither the culture filtrates nor the mycelial extracts had any specific toxicity at 40 μg dry weight per milliliter (Table 3). The difference in solids most likely was due to the presence of toxin in race T extracts; neither the *alb*1 or *MAT*A allele affected either the yields or specific activities of extracts (Tables 2 and 3).

Cultures of each of the two race O isolates were extracted four times. In several of these extracts a very slight precipitate was visible; the largest amount of precipitate (0.12 mg/flask) was present in an extract from isolate C8. The precipitate was bioassayed but caused only 5% inhibition of dark CO_2 fixation at 10 μg/ml on both Tms- and N-cytoplasm corn. All extracts from both race O isolates failed to show host-specific activity at concentrations as high as 80 μg/ml (Table 4).

DISCUSSION

The T-toxin purified from the race T isolates used in this study was chemically and biologically identical to the material Kono and Daly (8) identified as T-toxin. In our experience with several

TABLE 1. Inhibition of dark CO_2 fixation in Tms- and N-cytoplasm corn leaf slices by culture filtrates of four near-isogenic isolates of *Cochliobolus heterostrophus*

Filtrate source			Corn	Inhibition (%) in culture filtrates diluted:					
Isolate	Genotype	Race	cytoplasm	1/5,000	1/2,500	1/1,000	1/500	1/250	1/125
C4	*MAT*a, *ALB*1, *TOX*1	T	Tms	15	29	43	44	57	···
			N	5	4	9	5	10	···
C6	*MAT*A, *alb*1, *TOX*1	T	Tms	3	7	22	37	50	···
			N	2	−4[a]	4	3	7	···
C7	*MAT*A, *alb*1, *tox*1	O	Tms	···	···	···	−2	−2	0
			N	···	···	···	−3	−5	−5
C8	*MAT*a, *ALB*1, *tox*1	O	Tms	···	···	···	1	1	1
			N	···	···	···	1	2	6

[a] Values prefixed by a minus sign indicate a stimulation in the rate of dark CO_2 fixation.

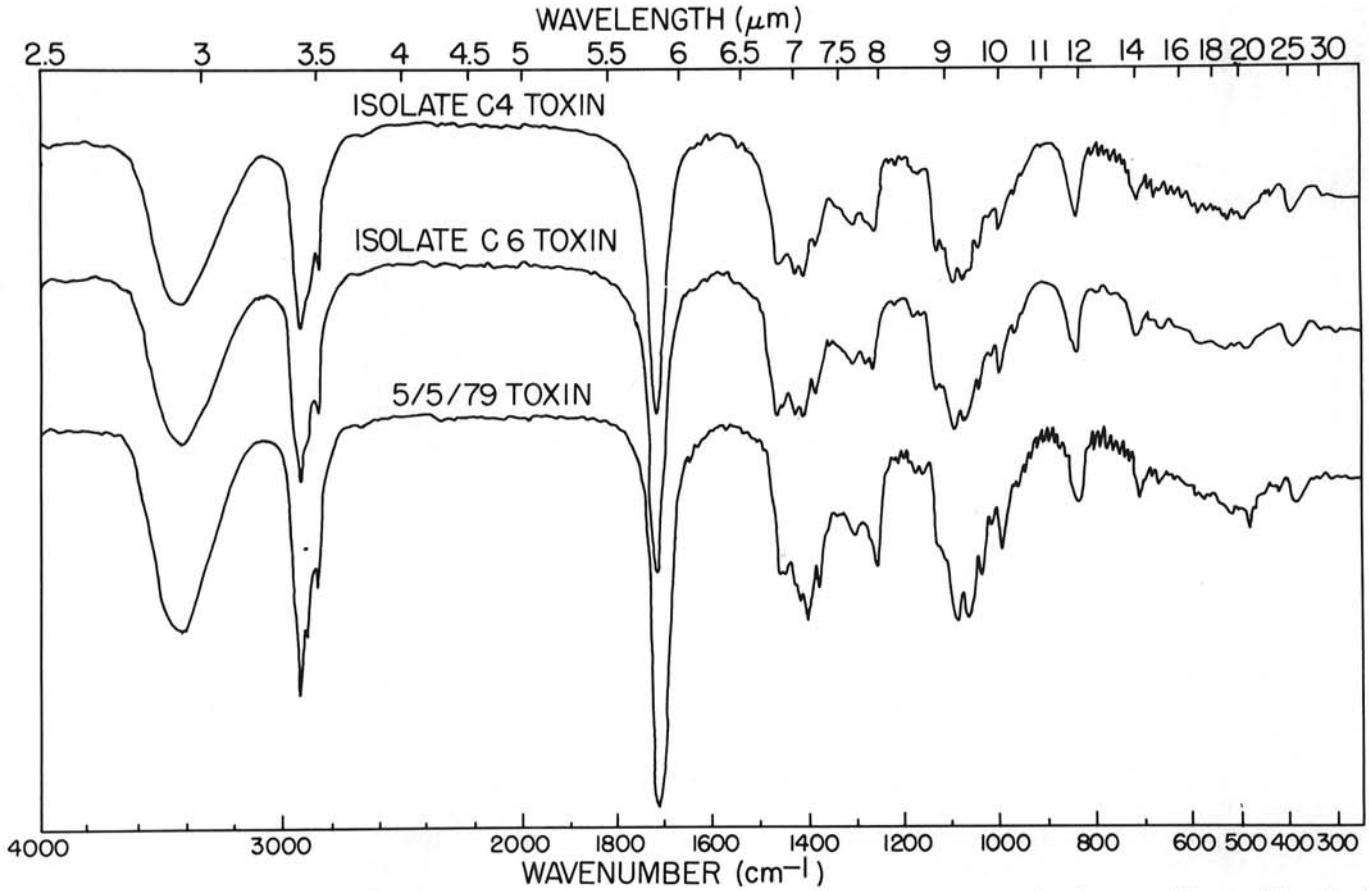

Fig. 1. Infrared spectra of toxin preparation of near-isogenic lines of *Cochliobolus heterostrophus* race T cultures and toxin prepared from a wild type strain (5/5/79 toxin).

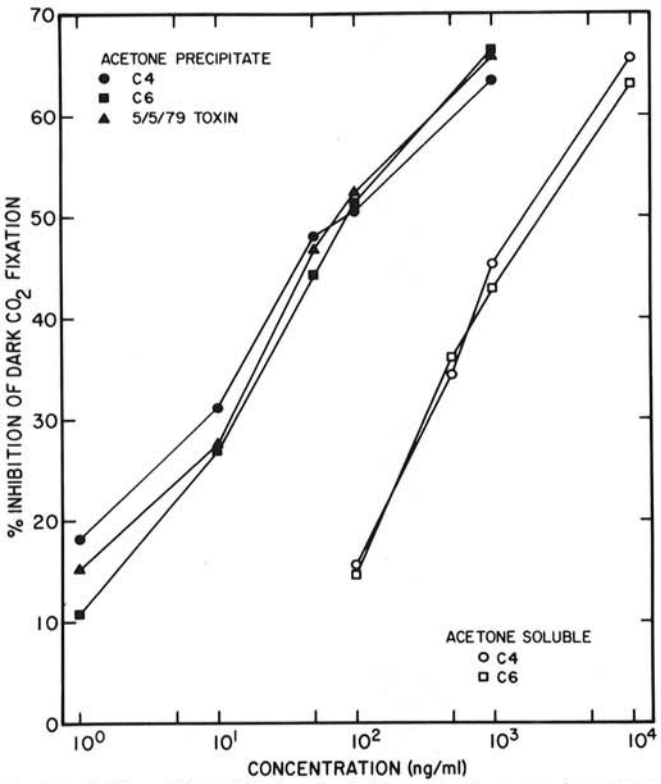

Fig. 2. Inhibition of dark CO_2 fixation in Tms-cytoplasm corn by acetone-insoluble and acetone-soluble materials from extraction of *Cochliobolus heterostrophus* race T cultures. Precipitates from isolates C4 (●) and C6 (■) are compared in activity to T-toxin preparation 5/5/79 (▲); activities of acetone-soluble material from isolates C4 (○) and C6 (□) are given. Weights of precipitates are the final concentrations in the assay solution.

TABLE 2. Yield of extracts from mycelia and culture filtrates from cultures of four near-isogenic isolates of *Cochliobolus heterostrophus* differing in ability to produce T-toxin

			Extract per flask[a] (mg)	
Isolate	Genotype	Race	Mycelium	Culture filtrate
C4	*MAT*a, *ALB*1, *TOX*1	T	121	34
C6	*MAT*A, *alb*1, *TOX*1	T	162	55
C7	*MAT*A, *alb*1, *tox*1	O	30	29
C8	*MAT*a, *ALB*1, *tox*1,	O	33	21

[a] Four flasks of each race T isolate and eight flasks of each race O isolate were used.

separate isolations, the albino phenotype did not affect the yield of toxin from the isolates, but it did lessen the amount of pigments present in the extracts, facilitating toxin purification. Analogous precipitable material was not detected in extracts from race O isolates. Since the isolates used in this study are near-isogenic, a fortuitous association between race of the isolate and presence of the precipitate seems unlikely. Therefore, the most probable identity of the precipitate is T-toxin. The apparently identical specific biological activity of these separate isolations (Fig. 2) also tends to eliminate an unknown contaminant as the host-specific toxicant.

From data presented in Tables 2 and 3, it can be calculated that over 95% of the T-toxin from race T isolates was recovered from the mycelium. Most of the previous research on the purification of toxin had been conducted by using culture filtrates, not mycelia, as the source of toxin. Our conclusion that extracts from the mycelium of race O contain no detectable T-toxin was possible only because experiments were done with glassware cleaned by following the precautions outlined by Payne et al (14) to remove traces of glass-bound toxin. In preliminary examinations, using

TABLE 3. Inhibition of dark CO_2 fixation by extracts from the mycelia and culture filtrates of near-isogenic isolates of races T and O of *Cochliobolus heterostrophus*

Isolate	Genotype	Race	Source	Extract concentration[a] (μg/ml)	Inhibition (%) in corn cytoplasm	
					Tms	N
C4	*MAT*a, *ALB*1, *TOX*1	T	Mycelium	1	69	14
			Culture filtrate	10	65	12
C6	*MAT*A, *alb*1, *TOX*1	T	Mycelium	1	61	11
			Culture filtrate	10	63	9
C7	*MAT*A, *alb*1, *tox*1	O	Mycelium	40	9	10
			Culture filtrate	40	11	2
C8	*MAT*a, *ALB*1, *tox*1	O	Mycelium	40	17	12
			Culture filtrate	40	8	11

[a] Final dry weight of extract per milliliter of assay solution.

TABLE 4. Inhibition of dark CO_2 fixation by extracts from cultures of two near-isogenic race O isolates of *Cochliobolus heterostrophus*

Isolate	Genotype	Corn cytoplasm	Inhibition (%) extract concentration (μg/ml)[a]			
			10	20	40	80
C7	*MAT*A, *alb*1, *tox*1	Tms	9	15	18	27
		N	14	16	19	30
C8	*MAT*a, *ALB*1, *tox*1	Tms	2	5	10	10
		N	2	7	13	19

[a] Final dry weight of extract per milliliter of assay solution.

glassware cleaned only by the usual laboratory procedures, traces of host-specific toxicity in race O cultures were noted. Subsequently, studies showed that sufficient toxin adheres to culture flasks, after routine washing, to cause such results. The misleading results obtained in our initial experiment emphasize the need for the precautions in cleaning glassware, especially when attempting to quantify T-toxin production.

In view of the apparent single-gene control of the race differences in these cultures and the nonessential nature of T-toxin accumulation for fungal growth, it is surprising to find that the *TOX*1 allele can cause up to 2% of the mycelial dry weight to be precipitable T-toxin (the percentage is actually greater than this because only a portion of the material exhibiting host-specific activity was precipitated from extracts). However, these results are in agreement with studies using a different isolate (*unpublished*). Further, T-toxin is not a single chemical species, but consists of eight to 10 species differing in chain length and replacement of a single carbonyl group by a hydroxyl group (8–10). Obviously, synthesis of T-toxin by race T represents a major metabolic event, which biochemically may be analogous to fatty acid biosynthesis. The fatty acid synthetase complex in *Saccharomyces cerevisiae* is under the control of at least three genes located on different chromosomes (11). In the present study, only a single locus with two alleles was examined. However, there is preliminary evidence that T-toxin may be under control of several loci (20).

The origin of race T is obscure. It has been hypothesized (19) that race T arose from race O through a spontaneous mutation that blocked a point in a normal metabolic pathway, thus leading to accumulation of an intermediate product (T-toxin). Our results neither support nor eliminate this possibility, nor do they exclude the possibility that race O synthesizes T-toxin but has additional enzyme(s) capable of further metabolizing it to other products. These questions can be answered only when biochemical details of the synthesis and metabolism of T-toxin are known.

To our knowledge this is the first report of a single genetic allele, essential for high virulence towards a host plant, which controls the synthesis of a chemically defined fungal product.

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