Host Specificity of a Toxin from Phyllosticta maydis for Texas Cytoplasmically Male-Sterile Maize

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ABSTRACT

Phyllosticta maydis produced, in culture, a host-specific toxin that caused plant responses similar to those caused by *Helminthosporium maydis* race T toxin. *P. maydis* toxin selectively inhibited seedling root growth, induced leaf chlorosis, and caused an increased leakage of electrolytes from maize leaves containing Texas male sterile cytoplasm (Tcms). Toxin treatment of mitochondria isolated from Tcms maize caused an immediate irreversible swelling, uncoupled oxidative phosphorylation, and (depending upon the substrate) either stimulated or inhibited O_2 uptake. The toxin did not affect maize with normal cytoplasm except at very high toxin concentrations.

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Yellow leaf blight of maize, caused by *Phyllosticta maydis* Arny & Nelson (1), was first reported in Wisconsin in 1967 (2). Since then, the disease has been observed in the cooler areas of the Corn Belt and northeastern USA (2). The characteristic disease symptoms are necrotic lesions with yellowing of surrounding tissue, and are similar to other diseases in which host-specific toxins are known to be involved.

The susceptibility of maize to *P. maydis* is partly controlled cytoplasmically. Maize lines vary in susceptibility to *P. maydis*, but inbred lines with Texas cytoplasmic male sterility (Tcms) are more susceptible than corresponding inbreds with normal cytoplasm (3). *Helminthosporium maydis* race T produces a toxin specific for Tcms and related maize cytoplasms (6); thus, *P. maydis* also might produce a Tcms-specific toxin. Our objectives were: (i) to evaluate whether or not *P. maydis* produces a host-specific toxin, (ii) to determine some biological responses to the toxins, and (iii) to compare the activity of *P. maydis* toxin with the activity of *H. maydis* race T toxin. O. C. Yoder, at Cornell University, has concurrently discovered a toxin produced by *P. maydis* (16). Preliminary reports have been published (4, 17).

MATERIALS AND METHODS.–Isolates of *P. maydis* were obtained from A. H. Epstein and D. C. Foley, and *H. maydis* race T was the Mitchellville isolate by C. A. Martinson, all from Iowa State University. Identities were confirmed by Lois H. Tiffany, Iowa State University.

Toxin was partly purified from culture filtrates of *P. maydis* by methods similar to those used for preparing the host-specific toxin produced by *H. maydis* race T (5). The fungus was grown 15 to 18 days in 250-ml Erlenmeyer flasks containing 50 ml of modified Fries' medium supplemented with 0.1% yeast extract (10). The filtrate was adjusted to pH 3.5

before 10-fold concentration in vacuo. Methanol (2:1, v/v) was added to the preparation, and it was stored overnight at 4 C. A precipitate was removed by filtration through Whatman No. 1 filter paper. Methanol was removed in vacuo, and the preparation was concentrated to 0.04 its original volume and had a dry weight of 164.5 mg/ml. This preparation was used in some experiments or was purified further by five extractions with equal volumes of ethyl acetate. The ethyl acetate fractions were combined, water was added, and the ethyl acetate was removed in vacuo. The final volume was adjusted to 0.04 of the original culture filtrate volume. Because the toxin preparation was impure, toxin activity depended on the volume or dilution of the preparation used and exact duplication with other preparations would not be expected.

Toxin activity was routinely assayed by injecting 0.2 ml of toxin solution into immature leaf whorl tissue (14); chlorosis developed within 3 days in toxin-sensitive plants. The following maize inbreds containing Tcms, Trf (Tcms with the male-fertility restoration gene), and normal cytoplasm were used: A632 Tcms, B37 Tcms, Oh43 Tcms, Wf9 Tcms, W64A Tcms, B37 Trf, Oh43 Trf, Wf9 Trf, A632, B37, Oh43, Wf9, and W64A. The concentration of toxin was determined by assaying a series of dilutions of the toxin preparation. Two plants of each maize inbred were used for each dilution and the experiment was repeated. The greatest dilution of toxin that induced chlorotic streaking in all the Tcms and Trf inbreds was defined as the dilution end point.

P. maydis toxin activity was determined using an inhibition of seedling root growth bioassay similar to that used to assay *H. maydis* race T and *H. carbonum* toxins (5, 13). Seeds were incubated, embryo side down, between moist paper towels for 48 hr. After which, seeds were selected for uniformity of root length 3.0 - 8.0 mm and five seeds were placed embryo side down in a 90-mm petri plate containing 10 ml of either White's solution or toxin diluted with White's solution. A series of toxin dilutions were assayed, and for each dilution duplicate petri plates containing a total of 10 seeds were used. After 3 days incubation in toxin at 24-26 C the roots were measured and compared to nontoxin-treated controls.

In the mitochondrial studies, seeds of normal and Tcms maize inbreds of W64A were germinated on paper toweling saturated with 0.1 mM $CaCl_2$ and grown in the dark (29 ± 1 C). Mitochondria were isolated from 3-day-old etiolated shoots according to the procedure of Miller et al. (8). The final mitochondrial pellet was suspended in 0.4 M sucrose. All experiments were carried out in a filled 4.0-ml, temperature-controlled (27 ± 0.2 C) glass reaction cell containing 200 mM KCl, 20 mM tris-HCl (pH 7.5), 5 mM KH_2PO_4 and 1 mg/ml bovine serum albumin. A Clark oxygen electrode (Yellow Springs Instrument Company) was fitted into the top of the cell and the cell assembly was placed in the light path of a Bausch & Lomb Spectronic 70 spectrophotometer. Cell contents were stirred with a magnetic stirrer. The rate of O_2 uptake (measured polarographically) and the rate of mitochondrial swelling (measured as the percentage of light transmission through the reaction cell) were recorded simultaneously on a dual channel recorder. Approximately 0.6 mg mitochondrial protein was used in each reaction. Mitochondrial protein concentrations were determined by the procedure of Lowry et al. (7) with bovine serum albumin standards. Other reaction components were as indicated in the table and figure legends.

The effect of toxin on electrolyte leakage was determined by monitoring the conductance of the ambient fluid of toxin-treated leaf tissue. Leaves from 12-day-old maize inbred (B37 Tcms and B37) seedlings were cut into 1-cm sections. Triplicate 1-gram samples of tissue were placed in cheese cloth bags, vacuum-infiltrated, and incubated in either a partly purified toxin solution or water for 4 hr on a reciprocal shaker (50-70 strokes/min). After which, the tissue was rinsed thoroughly, placed in 50 ml water/300-ml Erlenmeyer flask, and returned to the reciprocal shaker. Conductivity readings of the ambient fluid were made immediately and at 0.5, 1.0, 2.0, and 3.0 hr thereafter with a Yellow Springs conductivity bridge (cell constant k=1.0).

RESULTS.—*Sensitivity of immature leaf tissue of maize and various gramineous species to Phyllosticta maydis toxin.*—When toxin partly purified by ethyl acetate extraction was injected into immature leaf whorl tissue, chlorotic streaks developed on toxin-sensitive plant leaves within 3 days. Maize inbreds containing Tcms and Trf (Tcms with the male-fertility restoration gene) were the most sensitive to toxin. Chlorosis was induced in A632 Tcms, B37 Tcms, Oh43 Tcms, Wf9 Tcms, B37 Trf, Oh43 Trf, and Wf9 Trf inbreds treated with toxin diluted 1:1,000. Chlorosis did not develop in the normal-cytoplasm inbreds A632, B37, Oh43, Wf9, and W64A treated with toxin diluted only 1:25. Furthermore, other gramineous crop species (viz., millet, sudan grass, wheat, grain sorghum, barley, and oats), did not develop chlorosis at any dilution levels. A concentrated crude *P. maydis* toxin preparation gave comparable results, except that chlorosis was induced in maize by slightly higher dilutions. Thus, *P. maydis* toxin selectively induced chlorosis in maize leaves containing Tcms whether or not fertility was genetically restored.

Comparative inhibition of seedling root growth of maize containing Tcms and normal cytoplasm by Phyllosticta maydis toxin.—*P. maydis* toxin selectively inhibited the root growth of maize seedlings containing Texas male-sterile cytoplasm. Toxin preparations not purified by ethyl acetate extraction caused a 50% inhibition of root growth of maize containing Tcms (Wf9 Tcms and W64A Tcms) and normal cytoplasm (Wf9 and W64A) at dilutions of 1:1,000 and 1:25, respectively. This was a 40-fold greater sensitivity of maize with Tcms to this toxin preparation than from maize with normal cytoplasm.

Effect of toxin concentration on mitochondrial swelling and oxidation of NADH.—Mitochondria utilizing NADH as an electron source were treated with crude toxin at concentrations ranging from 0.1

μliter to 100 μliter/4.0 ml. The O_2 uptake rates for Tcms and normal mitochondria were comparable before toxin addition. However, the rate of O_2 uptake by normal mitochondria was not stimulated by the addition of toxin during the reaction, while the rate for Tcms mitochondria was stimulated substantially (Fig. 1). Tcms mitochondria showed a stimulation up to a concentration of 5 μliter/4.0 ml, the oxidation rate was reduced 25%. Although the rate of NADH oxidation by mitochondria isolated from normal W64A seedlings showed no stimulation by toxin, the O_2 uptake rate was reduced significantly at high toxin concentrations.

The toxin-induced swelling responses (Fig. 2) of Tcms and normal mitochondria were similar to the effects on O_2 uptake. Swelling was measured as the difference between the percent transmittance (% T) readings 3 min after and immediately before toxin addition. Swelling of Tcms mitochondria increased rapidly as a function of toxin concentration, reached a relatively stable level and then declined at the highest concentration. Normal mitochondria did not swell at low toxin concentrations; the only response was a decrease in % T (opposite of swelling) at the higher toxin levels.

The effects of toxin on mitochondria isolated from normal seedlings probably were caused by a high salt content of the toxin which, at high concentrations, precipitated mitochondrial protein and inhibited the electron transport system. This effect also was seen with Tcms mitochondria since swelling was reduced and NADH oxidation was inhibited instead of stimulated at high toxin levels. In similar studies with *H. maydis* race T toxin, high toxin concentrations did not reduce the effects of toxin on Tcms mitochondria (B. G. Gengenbach, *unpublished*). The discrepancy could have arisen from the different methods used to prepare the toxins or from the production of additional toxic compounds by *P. maydis*.

Toxin effects on O_2 uptake rates and oxidative phosphorylation.—Since the stimulation of Tcms NADH oxidation reached a maximum at the 5 μliter/4.0 ml toxin concentration (Fig. 1), subsequent mitochondrial work was done with this concentration. In the absence of toxin, respiration of Tcms or normal mitochondria utilizing any of the three substrates was increased to the state 3 control rates by the addition of ADP (Table 1). When the added ADP was depleted, lower state 4 control rates were maintained. The control ADP/O ratios indicated that oxidative phosphorylation was coupled to electron transport in both normal and Tcms mitochondria.

Subsequent addition of toxin to the reaction vessel altered the state 4 respiration rates of Tcms mitochondria (Table 1). The oxidation rates of malate + pyruvate and succinate were inhibited and, as previously indicated, NADH oxidation was stimulated. When ADP was then added after toxin, no stimulation of the O_2 uptake rate (toxin state 3) over the previous rate (toxin state 4) was obtained. The lack of stimulation indicated that the Tcms mitochondria were not capable of phosphorylation after toxin treatment; the resultant ADP/O ratios were zero. In contrast, toxin had little effect on state 4 and state 3 O_2 uptake rates and ADP/O ratios of normal mitochondria. These effects of *P. maydis* toxin were very similar to the effects of *H. maydis*

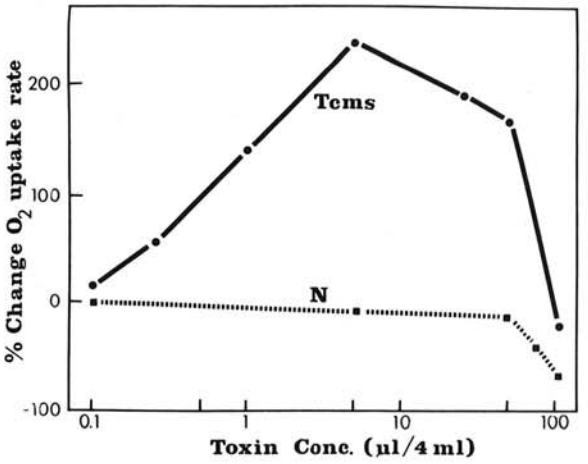

Fig. 1. Rate of NADH oxidation by isolated Tcms and normal maize mitochondria treated with various concentrations of *Phyllosticta maydis* toxin. The reaction medium (4 ml) contained 200 mM KCl, 20 mM tris-HCl, 4 mM KH_2PO_4, and 1 mg/ml bovine serum albumin. Respiration was started by adding 2 μmoles NADH. A base O_2 uptake rate was established, then the indicated volume of toxin was added. The percent change was calculated from O_2 uptake rates before and 3 min after toxin addition. Each point is the mean of two replicates.

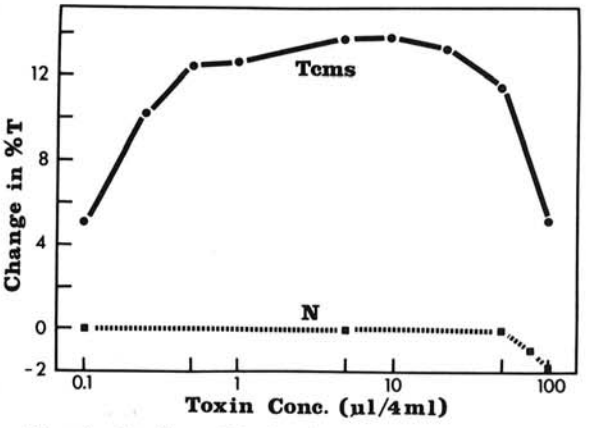

Fig. 2. Swelling of isolated Tcms and normal maize mitochondria, treated with *Phyllosticta maydis* toxin. The reaction medium (4 ml) contained 200 mM KCl, 20 mM tris-HCl, 4 mM KH_2PO_4, and 1 mg/ml bovine serum albumin. Mitochondria were added and allowed to swell passively for 2 min. Swelling stopped upon addition of 2 μmoles NADH and the percent transmittance (%T) of light through the suspension was constant. Toxin was then added and swelling was measured as the increase in % T that occurred during the following 3 min. Each point is the mean of two replicates and is expressed as change in %T/3 min/mg protein.

TABLE 1. Effects of *Phyllosticta maydis* toxin on mitochondrial respiration and oxidative phosphorylation.

The reaction medium (4 ml) contained 200 mM KCl, 20 mM tris-HCl, 4 mM KH_2PO_4 and 1 mg/ml bovine serum albumin. Respiration was initiated by the addition of either 40 μmoles malate + 40 μmoles pyruvate, 40 μmoles succinate, or 2 μmoles NADH. The order of subsequent additions was 300 nmoles ADP, 5 μliter toxin, and 300 nmoles ADP at 2- to 3-min intervals. Control rates and ADP/O ratios were obtained from recorder traces before toxin addition; toxin rates and ADP/O ratios were obtained after toxin addition. Values are the mean of two replicates

		nmoles O_2/min/mg protein					
		State 4		State 3		ADP/O	
Cytoplasm[a]	Substrate	Control	Toxin	Control	Toxin	Control	Toxin
N	Malate + Pyruvate	35	27(−27)[b]	84	64(−24)[b]	1.68	1.84
Tcms	Malate + Pyruvate	27	0(−100)	94	0(−100)	2.05	0
N	Succinate	91	93(+2)	152	169(+9)	1.13	1.33
Tcms	Succinate	85	36(−58)	171	36(−79)	1.31	0
N	NADH	85	79(−7)	269	245(−9)	1.18	1.15
Tcms	NADH	66	234(+244)	197	234(+19)	1.20	0

[a] N = normal (male fertile) cytoplasm; Tcms = Texas cytoplasm, male sterile.

[b] The values in parentheses indicate the percent inhibition or stimulation of the toxin rate compared to the control rate.

toxin on isolated normal and Tcms mitochondria reported previously (9).

The effect of Phyllosticta maydis toxin on electrolyte leakage from corn leaves.—There was a large increase in electrolyte loss from toxin-treated Tcms leaves compared with water-treated controls (Fig. 3). In contrast, there was only a slight increase as a result of toxin treatment in loss from leaves containing normal cytoplasm. The experiment was repeated with comparable results. Whether or not the effect on tissue with normal cytoplasm was due to toxin or impurities in the preparation is not known.

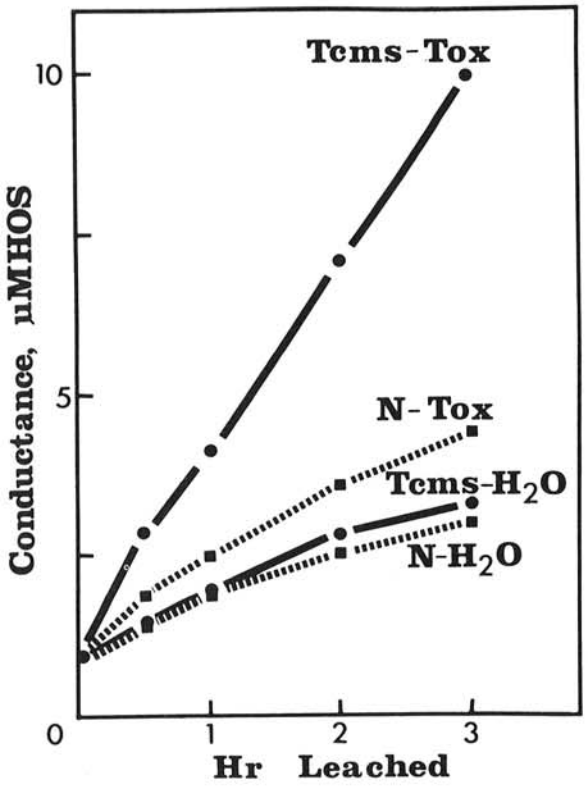

Fig. 3. Loss of electrolytes from Tcms and normal maize leaves treated with *Phyllosticta maydis* toxin.

DISCUSSION.—The host-selective toxins produced by *H. maydis* race T and *P. maydis* have similar activities toward maize containing Tcms. When tested on Tcms maize, the toxin from *P. maydis* inhibited seedling root growth, induced chlorosis and electrolyte leakage, and disrupted normal functions of isolated mitochondria. A similar spectra of phenomena have been observed on Tcms maize with *H. maydis* race T toxin (6, 9, 14). In addition, the percentages for stimulation and inhibition of mitochondrial state 4, O_2 uptake rates by *P. maydis* toxin (Table 1) agreed closely with those for *H. maydis* race T toxin treatment of the same inbred (B. G. Gengenbach, *unpublished*). Maize with normal cytoplasm was not affected by the same toxin treatments.

The results indicate that the mitochondria from Tcms maize have a site on which *P. maydis* and *H. maydis* race T toxins act. None of the other host-specific toxins that have been examined affect the mitochondria isolated from their respective susceptible hosts (12, 15).

The similar activities of the two toxins suggest that they are the same or closely related chemically and/or that they have a similar primary site of action. Differences in their sites and modes of action have not been found. Clarification of these points awaits determination of their structures and primary sites of action.

Genetically, male sterility and sensitivity of *P. maydis* and *H. maydis* race T toxins are both cytoplasmically controlled, indicating that these two phenomena are controlled by either two closely linked plasma genes or are different manifestations of the same plasma gene. It appears that the wide use of Tcms maize has put selection pressure on these two

unrelated pathogens such that the organisms now produce toxic metabolites specific for this maize.

There is not a complete correlation between the sensitivity to *P. maydis* toxin and susceptibility to the fungus. Susceptibility of maize to *P. maydis* is genetically and cytoplasmically controlled (3) but sensitivity to *P. maydis* toxin is only cytoplasmically controlled. This suggests that other factor(s) in addition to the host-specific toxin described in this paper are involved in pathogenesis. Yoder & Mukunya (17) have reported that *P. maydis* culture filtrates contained host-specific toxic metabolite(s) having the same specificity as the pathogen. If this is the case, our results indicate that another host-specific metabolite other than the Tcms selective toxin is also involved in pathogenesis. At least one other pathogen, *Periconia circinata* is known to produce more than one host-specific toxin, but they have the same specificity (11). Our failure to extract toxic metabolites correlating completely to the pathogenicity of *P. maydis* may be due to the *P. maydis* isolates, methods of toxin production and extraction procedures used. Further research is required to clarify whether or not *P. maydis* produces a host-specific metabolite(s) other than the Tcms specific toxin.

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