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thesis entitled

EFFECTS OF THE HOST-SELECTIVE TOXIN FROM

PHYLLOSTICTA MAYDIS ON T-CYTOPLASM CORN

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Plant Physiology

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Date Feb. 13, 1980

EFFECTS OF THE HOST-SELECTIVE TOXIN FROM *PHYLLOSTICTA MAYDIS* ON T-CYTOPLASM CORN

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1980

ABSTRACT

EFFECTS OF THE HOST-SELECTIVE TOXIN FROM *PHYLLOSTICTA MAYDIS* ON T-CYTOPLASM CORN

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A host-selective toxin was partially purified from liquid cultures of *Phyllosticta maydis*. The toxin (*P.maydis* toxin) caused various physiological changes in corn with the Texas male sterile genes (T-cytoplasm) whereas corn with normal (N) cytoplasm was not affected. The toxin, at a concentration of 4.7 µg/ml, inhibited root growth in susceptible seedlings by 50%. This assay was unsatisfactory because of the wide variation within each toxin treatment. The toxin also inhibited CO_2 fixation in the dark by leaf disks from T-cytoplasm corn; 2.6 µg of toxin per ml inhibited CO_2 fixation by 50%.

P.maydis toxin induces various changes in mitochondria isolated from T-cytoplasm corn tissues. These effects varied with the exogenous substrate. The toxin stimulated NADH respiration, inhibited malate+pyruvate respiration, and partially inhibited succinate respiration. In all cases, these effects were seen only in mitochondria from T-cytoplasm corn tissues; mitochondria from N-cytoplasm corn were insensitive to the toxin. The results with mitochondria confirm those reported previously.

The data show that the toxins from *Helminthosporium maydis* race T and *P.maydis* have similar effects on T-cytoplasm corn and on mitochondria isolated from such corn.

To my parents

Edward and Barbara

ACKNOWLEDGMENTS

The author wishes to express appreciation for the patient help and kind suggestions given by Dr. Robert P. Scheffer.

Further thanks are due to the other members of my guidance committee, Dr. N.E. Good and Dr. C.J. Pollard, for their advice and evaluation of this manuscript.

Finally, a special thanks to my wife Coleen whose understanding and friendship has helped me through the difficult parts of this project.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
Ci	Curie
DNP	2,4-dinitrophenol
ED_{50}	toxin concentration which causes 50% inhibition of some physiological process
HmT toxin	*Helminthosporium maydis* race T toxin
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
M	molar (concentration)
N	normal (concentration)
N	Normal or nonsterile (cytoplasm)
NAD	β-nicotinamide adenine dinucleotide, oxidized form
NADH	β-nicotinamide adenine dinucleotide, reduced form (disodium salt)
Pi	orthophosphate
P.maydis toxin	*Phyllosticta maydis* toxin
P/O	ratio of ATP formed to atoms of oxygen consumed
T	Texas male sterile (cytoplasm)

INTRODUCTION

Several plant pathogens produce metabolites which are toxic to their respective hosts; resistant cultivars and all non-hosts are insensitive to the toxic chemicals (27). These so-called host-selective toxins appear to determine host specificity.

Helminthosporium maydis race T, the cause of southern corn leaf blight, is an example of a fungal pathogen which produces a host-selective toxin (HmT toxin). Corn with the cytoplasmically-inherited genes for male serility (T-cytoplasm) are susceptible to the pathogen and sensitive to the HmT toxin; corn with normal (N) cytoplasm is resistant to the pathogen and insensitive to the toxin (15,17). Widespread use of corn with T-cytoplasm led to the disastrous epidemic of southern corn leaf blight in 1970 (28).

The genetic control of sensitivity to HmT toxin is not unique. *Phyllosticta maydis* Arny and Nelson, the causal pathogen of yellow leaf blight of corn, also produces a host-selective toxin (*P.maydis* toxin) specific for corn with T-cytoplasm (11,30). As with *H.maydis* race T and HmT toxin, the sensitivity of T-cytoplasm corn to *P.maydis* toxin determines the high degree of susceptibility to the pathogen. N-cytoplasm corn is insensitive to the toxin and resistant to the fungus.

Other workers (11, 30) have suggested that the toxins produced by *P.maydis* and *H.maydis* race T may be similar. These suggestions were made on the basis of the specific genetic control of sensitivity to the toxins (i.e., T-cytoplasm genes), and on limited studies showing

the physiological processes affected by the two toxins (11). My work has been to further characterize the physiological effects of *P.maydis* toxin on T-cytoplasm corn. The similarity in responces of sensitive corn to HmT and *P.maydis* toxins suggests that the toxins may have similar sites and modes of action. This would be expected if the two toxins were the same chemically. Final proof of chemical similarity must await the determination of the structures of HmT and *P.maydis* toxins.

LITERATURE REVIEW

Host-selective toxins and their relationship to disease

There are many plant diseases in which host-selective toxins are known to play an important role (27). The relationship between toxin and sensitive cells has proved valuable in the study of the molecular basis of disease development in plants. In all such cases, susceptibility to the pathogen is related to sensitivity to the toxin it produces. Resistance is determined by the insensitivity of the hosts to the toxins (27).

The diseases involving several known host-selective toxins appeared when new and uniform genotypes were widely grown (27). A classic example of this is the *Helminthosporium* blight of oats. A gene for resistance to crown rust (caused by *Puccinia coronata*) was used in several new oat cultivars. Unfortunately, these new cultivars were devistated by a new disease. The causal fungus (*H. victoriae*), was isolated and a host-selective toxin ("victorin") identified. The gene used for resistance to crown rust also gave special sensitivity to victorin.

A similar story exists for southern corn leaf blight. Plant breeders incorporated the cytoplasmically-inherited Texas male sterile (T-cytoplasm) gene into most corn hybrids. A new race of *Helminthosporium maydis* (race T) developed into a serious pathogen and caused severe losses in corn with the T-cytoplasm gene. The special path-

ogenicity of the fungus is based on its ability to produce a host-selective toxin (HmT toxin) specific for corn with the T-cytoplasm gene.

Phyllosticta maydis appears to have responded to the same selective pressures. It too produces a toxin selective for corn with T-cytoplasm. As with *H.maydis* and HmT toxin, susceptibility to the pathogen is correlated with sensitivity to the toxin, whereas resistance corresponds to insensitivity to the toxin.

Yellow leaf blight of corn and the *P.maydis* toxin

Yellow leaf blight is a relatively new disease of corn, first observed in Ohio in 1965 (20). The disease was soon found in Wisconsin and throughout the cooler corn growing regions of the United States and Canada (4). The disease is now widespread, but losses are not usually severe. However, local epiphytotics occasionally cause up to 50% loss in yield (4). Arny and Nelson (5) identified the causal organism of yellow leaf blight and described it as a new species: *Phyllosticta maydis*. The sexual stage of the fungus is called *Mycosphaerella zeae-maydis* (Mukunya and Boothroyd) (23).

Ayers *et al.* (6) evaluated numerous corn lines for susceptibility to *P.maydis*. They found that corn lines vary in susceptibility, but noticed that inbred lines with the cytoplasmically inherited Texas male sterile genes were always more susceptible than the same inbred with normal (N) cytoplasm. Nuclear genes may contribute somewhat to the susceptibility of the corn lines to *P.maydis*, but the major genetic factor controlling susceptibility is carried with the cytoplasmically inherited genes for male ster-

ility (6).

A host-selective toxin from *P.maydis* cultures was reported concurrently by Comstock *et al.* (11) and Yoder (30), who found that the fungus produced a metabolite toxic to corn which contained T-cytoplasm. All corn cultivars tested which contained T-cytoplasm were susceptible to the fungus and sensitive to the toxin (30). The corn cultivars with N-cytoplasm, and several other gramineous crops, were resistant to the fungus and insensitive to the toxin (11, 30).

Comstock *et al.* (11) reported several physiological effects of *P.maydis* toxin on T-cytoplasm corn. The toxin inhibited seedling root growth, induced electrolyte leakage, and disrupted the normal function of isolated mitochondria. These effects on mitochondria included immediate swelling and uncoupling of oxidative phosphorylation. When NADH was used as the substrate, the toxin stimulated oxygen uptake; when malate+pyruvate or succinate was used, the toxin inhibited oxygen uptake. Since some mitochondrial characteristics are inherited cytoplasmically, as is the control of sensitivity to *P.maydis* toxin, Comstock *et al.* (11) suggested that the mitochondrion from T-cytoplasm corn contained a site of action for *P.maydis* toxin.

Physiological effects of HmT toxin on T-cytoplasm corn

Many physiological effects of HmT toxin on sensitivite (T-cytoplasm) corn are known and have been discussed in a recent review (15). These include chlorosis and lesion formation in intact plants (14), inhibition of seedling root growth (17), and inhibition of CO_2 fixation in the dark (9). HmT toxin also disrupts the respiratory

activities of mitochondria isolated from T-cytoplasm corn; the effects include uncoupling of oxidative phosphorylation, stimulation of NADH oxidation, inhibition of malate+pyruvate oxidation, and partial inhibition of succinate oxidation (7,12,22). The toxin also induces swelling of mitochondria (13,22) and disrupts the inner mitochondrial membrane (12). Furthermore, toxin causes increases in respiration of intact tissues (8). The ultrastructural data supports the hypothesis that the mitochondrion is a site of action for HmT toxin *in vivo* (1,19). The cytoplasmic inheritance of susceptibility to the southern corn leaf blight disease and sensitivity to the HmT toxin is consistant with a mitochondrial site of action.

Toxic effects on mitochondria are substrate-dependent; this should aid in determining the exact mode of toxin action. The stimulation of NADH respiration by toxin in mitochondria from T-cytoplasm corn is probably caused by the uncoupling action of the toxin (7, 16). Toxin is known to inhibit malate+pyruvate respiration, the nature of which has been the subject of research. There is evidence that the toxin causes a leakage of endogenous NAD from the mitochondrion (16). The various effects of toxin on mitochondria might be caused by a single toxin/mitochondrion interaction. Perhaps all the effects of toxin on sensitive tissues can be traced to the effects on sensitive mitochondria.

MATERIALS AND METHODS

Corn plants

An inbred corn line, W64A, was used for all experiments. The inbred with Texas male sterile cytoplasm (T-cytoplasm) was used because it is sensitive to *P.maydis* toxin. The same inbred with normal (N) cytoplasm was used because it is insensitive to the toxin (17).

In experiments requiring green plant material, seeds were planted in flats containing potting soil. Seedlings were grown in the greenhouse and fertilized every four days with a soluble fertilizer. The third or fourth true leaf from 21-day old plants was used for CO_2-fixation experiments. For root growth bioassays, seeds were treated with Captan and placed embryo side down on wet filter paper. After incubation for 24 hours at 32 C, seedlings for assays were chosen for uniform root growth (about 2-3 mm).

Etiolated corn tissues were used as a source of mitochondria. Seedlings were grown in the dark in pans of vermiculite watered with White's nutrient solution (10). Shoots were harvested after 7 days at 21-23 C.

Fungal cultures

Various fungal isolates were assayed for toxin production and two were chosen for these experiments. Isolate Pm24 was from infected

corn grown in Michigan. Isolate TS40 was a temperature-sensitive mutant isolated by Dean Gabriel at Michigan State University; this was the best toxin producer among the isolates tested. All cultures were maintained on potato dextrose agar (PDA) slants and stored under refrigeration. Ten days before liquid medium was to be inoculated, petri dishes containing PDA were inoculated from stock sultures. The liquid medium was inoculated from these plates.

Toxin production

P.maydis cultures were frown for 14-21 days in Roux flasks at 21-23 C. Each flask contained 200 ml of Fries' solution (26) supplemented with 0.1% yeast extract (w/v). Cultures were harvested by filtration through 8 layers of cheesecloth followed by filtration with Whatman no. 1 paper. The culture filtrate was adjusted to pH 3.5 with HCl and concentrated *in vacuo* at temperatures below 35 C to 6 per cent of its original volume. Two volumes of methanol were added and the solution was stored overnight in the refrigerator. The resulting precipitate was removed by filtration and the methanol was then removed under reduced pressure. This aqueous concentrate was extracted three times with equal volumes of chloroform. The chloroform fractions were pooled, reduced to dryness, and dissolved in 100 ml of water. The chloroform extract was stored at 4 C; toxin activity was not lost after storage for many months.

Root growth bioassays

A seedling root growth bioassay was one of the means of measuring toxin activity. Corn seedlings were treated with Captan and germinated as previously described. Five seeds were placed in a 9 cm petri

dish containing 10 ml of toxin solution; a series of toxin dilutions were used. Control seeds were held in distilled water; resistant seedlings treated with toxin were used as controls in all assays. The seedlings were incubated in the dark for 72 hours at room temperature, after which root lengths were measured and an ED_{50} value was calculated.

CO_2-fixation bioassay

The ability of *P.maydis* toxin to inhibit the fixation of CO_2 in the dark was measured by a method previously described for HmT toxin (9). Leaf disks (5mm diameter) were cut from the third or fourth true leaves with a corn borer. Random samples of these disks were transfered to steel planchets each containing 1.8 ml of solution. KH_2PO_4 solutions (10 mM) were used in control planchets and for dilution of the toxin preparations. Leaf disks (10 per treatment) were pre-incubated in the presence of the test solutions for 4 hours under fluorescent lights at 21-23 C. The planchets were then placed in an airtight container (125 x 30 mm) which served as a CO_2-tagging chamber.

A dish containing 3 ml of 3 $\underline{N}$ H_2SO_4 was placed in the center of the chamber which was then sealed. A 1 ml aliquot (10 µCi) of $Na^{14}CO_3$ was injected into the acid through a serum stopper and the gas was evenly distributed in the chamber by pumping with a large syringe. The chamber was covered with aluminum foil to exclude light. The leaf disks were incubated for 2 hours in the presence of $^{14}CO_2$, after which they were transfered to scintillation vials containing 0.5 ml of digestion mixture (60% perchloric acid; 30%

H_2O_2, 1:1,v/v). The vials were capped and kept at 90 C for 1 hour. After cooling, 10 ml of scintillation fluid was added to each vial. Scintillation fluid was a mixture of 15 g Omnifluor (New England Nuclear Co.), 1 liter toluene and 1 liter Triton X-100. Samples were counted on a Beckman LS 133 liquid scintillation counter, and an ED_{50} value for inhibition was calculated. Leaf disks from N-cytoplasm corn were used as controls in all experiments.

Mitochondria

Mitochondria were isolated from 1 week old etiolated shoots grown as previously described. Whole corn plants were placed at 4 C for 4 hours before shoots (75 g) were cut into 1 cm pieces and ground (without sand) for 1 minute with a cold mortar and pestle. The entire procedure was carried out at <4 C and all samples and solutions were kept on ice. The grinding medium (150 ml) contained 0.4 M sucrose, 0.03 M Hepes-NaOH (pH 7.5), 5 mM EDTA, 0.05 per cent cysteine, and 0.1 per cent bovine serum albumin. The homogenate was filtered through eight layers of cheesecloth and centrifuged for five minutes at 28,000x g. A camel hair brush was used to resuspend the pellet in 30 ml of a modified grinding medium (same as the grinding medium but minus cysteine). The resuspended sample was centrifuged for 3 minutes at 2,500x g. The resulting mitochondrial pellet was suspended in 3.0 ml of a solution containing 0.4 M sucrose and 30 mM Hepes-NaOH (pH 7.5).

Mitochondrial activity was measured as oxygen consumption using a Clark-type oxygen electrode (Yellow Springs Instrument Co.) covered with a teflon membrane. The signal from the electrode was amplified

and fed into a recorder. A 3 ml aliquot of a reaction medium containing 0.2 M KCl, 0.2 M Hepes-NaOH (pH 7.5), 2 mM $MgCl_2$, 2.5 mM K_2HPO_4 and 0.3 ml of suspended mitochondria (containing 0.3-0.6 mg mitochondrial protein). Bovine serum albumin (0.1 per cent) was used in some experiments but caused foaming which made it difficult to exclude air from the solution in the reaction chamber. A magnetic stirrer continuously stirred the reaction medium which was held at 28 C by a controlled temperature bath. The oxygen electrode holder was equipped with a narrow groove which allowed the insertion of a three-inch long syringe needle. By this means, substrates and toxin were introduced into the reaction medium.

Mitochondria and the reaction medium were combined in the reaction chamber, and the oxygen electrode was inserted carefully to exclude air from the mixture. One of three sustrates was then added. They included NADH (0.5 mM), succinate (9 mM), and malate+ pyruvate (10 mM each). The substrates were added as 20 µl aliquots to avoid a significant change in the reagent concentration in the reaction mixture. Oxygen consumption was followed until a stable nonphosphorylating (state IV) respiration rate was obtained (usually 2-3 minutes). In some experiments, 20 µl of toxin was added after the nonphosphorylating rate was obtained and the toxic effects on respiration were monitored for 2-3 minutes. In other experiments, ADP (150 µM) was added and a stable phosphorylating (state III) respiration rate was obtained and followed for 2-3 minutes. Toxin was then added and changes in the phosphorylating rate were followed.

Respiration rates were calculated as nmoles oxygen consumed per

minute per mg mitochondrial protein. Mitochondrial protein was determined by the method of Lowry *et al.* (18b). This standardized the results to compensate for differences in the amounts of mitochondrial protein from one experiment to another. Toxin-induced changes are usually given as per cent stimulation or inhibition. This refers to the increase or decrease in the rate of oxygen consumption as compared to the respiration rate before the addition of toxin.

RESULTS

Effects of *P.maydis* toxin on seedling root growth

Root lengths from toxin-treated seedlings were compared with roots from seedlings grown in water without toxin to calculate per cent inhibition. Standard assay prodeedures were used. The activity of each toxin preparation was determined as the ED_{50} value; i.e., the concentration of toxin which caused 50% inhibition of seedling root growth.

Results from a representative experiment are given in Figure 1. In this experiment, seedlings of T-cytoplasm corn were inhibited 86% by 112 µg of toxin per ml. Resistant seedlings were not inhibited by the same concentration of toxin. Thus, *P.maydis* toxin inhibited root growth in pregerminated seedlings of T but not of N-cytoplasm corn. However, there was large variation in root lengths in the toxin treated and control samples. This was due in part to the use of inbred seeds of poor quality, which did not grow uniforly. However, if the mean root lengths of >20 seedlings were used, ED_{50} values could be accurately reproduced. The ED_{50} value of the toxin preparation reported in Fig. 1 was 4.7 µg of toxin per ml. Comparable results were obtained with several other toxin preparations. I concluded that root growth inhibition assays using inbred corn lines are not suitable for quantitative work.

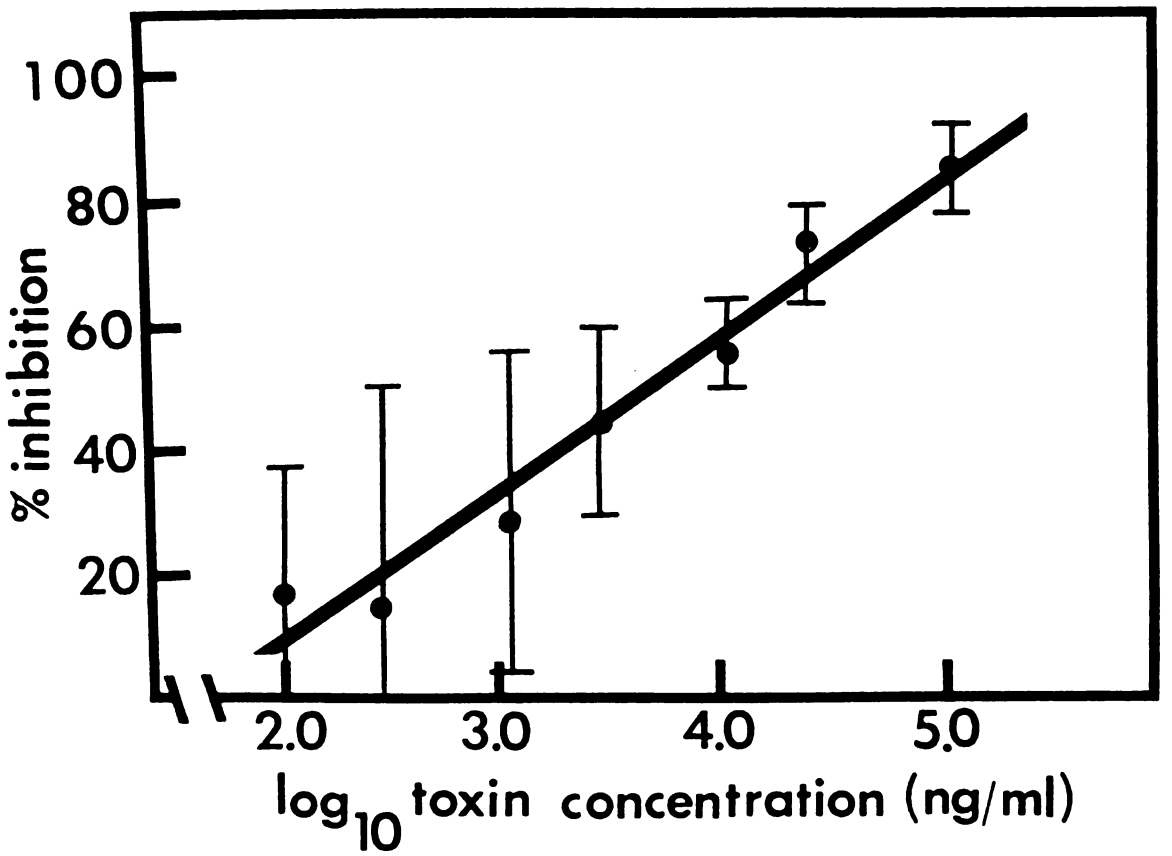

Figure 1. Effects of *Phyllosticta maydis* toxin on root growth by corn with T-cytoplasm. Seeds were pre-germinated and exposed to various concentrations of *P.maydis* toxin for 72 hours. Root lengths from toxin-treated seedlings were compared with root lenghts from control seedlings, and per cent inhibition was calculated. Each data point represents the mean inhibition of 20 roots in the toxin treated samples; variation (± one standard deviation) also is given. The control seedlings grew an average of 61 mm. The ED_{50} of this toxin preparation was 4.7 µg/ml.

Effects of *P.maydis* toxin
on CO_2-fixation

Corn leaf disks prepared as described previously were floated on a solution containing KH_2PO_4 (10 mM), plus toxin at various concentrations. Control leaf disks were floated on KH_2PO_4 solutions without toxin. The leaf disks were incubated in the toxin solutions in the light for 4 hours and exposed to $^{14}CO_2$ for 2 hours in the dark. They were then digested and the radioactivity was counted. Samples exposed to toxin were compared to the controls without toxin and per cent inhibition of CO_2 uptake by the toxin was calculated as with the root growth bioassay.

Results from a representative CO_2-fixation experiment, using partially purified toxin, are given in Figure 2. *P.maydis* toxin at a concentration of 2.6 µg/ml caused 50% inhibition of CO_2-fixation in susceptible (T-cytoplasm) leaf disks. N-cytoplasm corn was not affected by toxin at 28 µg/ml. The data from this and many other such assays show that CO_2-fixation in leaf disks from T-cytoplasm corn is inhibited by *P.maydis* toxin. Leaf disks from N-cytoplasm corn were not affected by more than 100 times higher concentrations of the toxin. Effects of toxin on CO_2 uptake in the dark was routinely used as an assay to measure toxin activity.

Effects of *P.maydis* toxin
on isolated mitochondria

Other workers have shown that HmT toxin causes rapid, host-selective changes in mitochondria isolated from corn (22). Uncoupling of oxidative phosphorylation is evident (7). This effect was correlated with a rapid increase in oxygen consumption when NADH

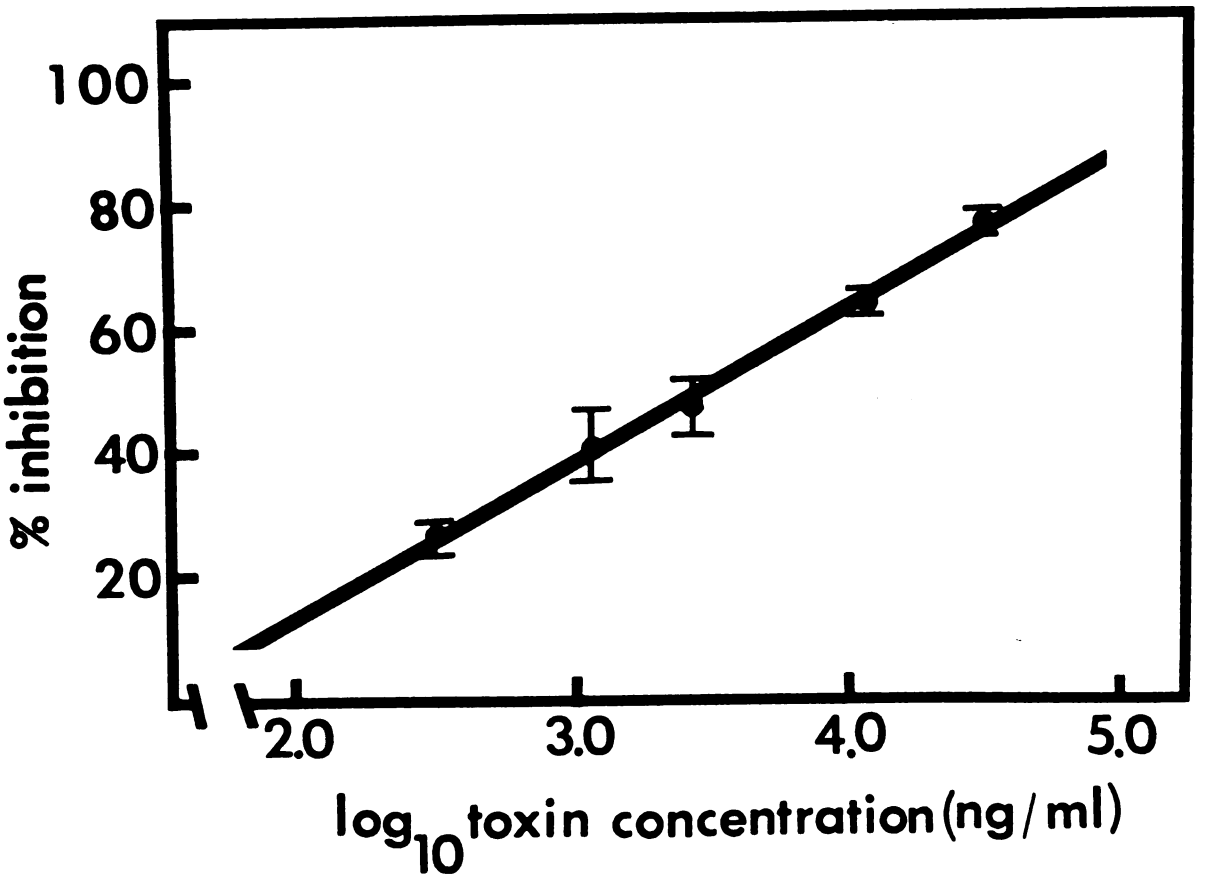

Figure 2. Effects of *Phyllosticta maydis* toxin on CO_2 fixation in the dark by tissues from T-cytoplasm corn. Leaf disks from T and N-cytoplasm corn were exposed to solutions containing various concentrations of *P.maydis* toxin for 4 hours in the light. The leaf disks were exposed to $^{14}CO_2$ for 2 hours in the dark. The leaf disks were then digested and the radioactivity was counted. Counts per minute from toxin treated samples were compared with counts for controls without toxin, which averaged 1424 counts per minute. Each data point represents the mean of 2 or 3 toxin treated samples; maximum and minimum values for each treatment are given. The toxin preparation was the same as used in the root growth experiment (Fig. 1). ED_{50} in this CO_2 experiment was at 2.6 µg toxin per ml.

was the exogenous substrate (22). Oxygen consumption was affected differently when other substrates were used. I have completed similar experiments with *P.maydis* toxin.

Respiratory control rates (RCR) were used as a measure of the degree of coupling in mitochondrial preparations. The RCR was calculated from the increase in oxygen consumption when the nonphosphorylating rate was compared with the phosphorylating rate obtained after the addition of ADP. If the addition of ADP doubled the rate of oxygen consumption (RCR = 2.0), the mitochondria were considered well coupled and the preparation was used in experiments. Mitochondrial preparations with a RCR of less than 2.0 were not used.

An aliquot of a well-coupled mitochondrial preparation was added to a reaction medium containing Pi but without ADP and substrate. NADH was added to establish a nonphosphorylating (state IV) respiration rate. After 2-3 minutes, *P.maydis* toxin was added. The toxin stimulated oxygen consumption by T but not by N-cytoplasm mitochondria when NADH was the substrate.

A respresentative oxygen electrode trace showing the effects of *P.maydis* toxin on NADH oxidation in susceptible mitochondria is shown in Figure 3. The rate of oxygen consumption is represented by the slope of the trace; steeper slopes correspond to more rapid rates of oxygen consumption. This trace shows the results from a single experiment, but similar results were obtained in all of many experiments.

Stimulation of NADH-induced oxygen consumption was found to be dose dependent (Figure 4). In this experiment, various amounts of toxin were added to the mitochondria in the reaction medium and the

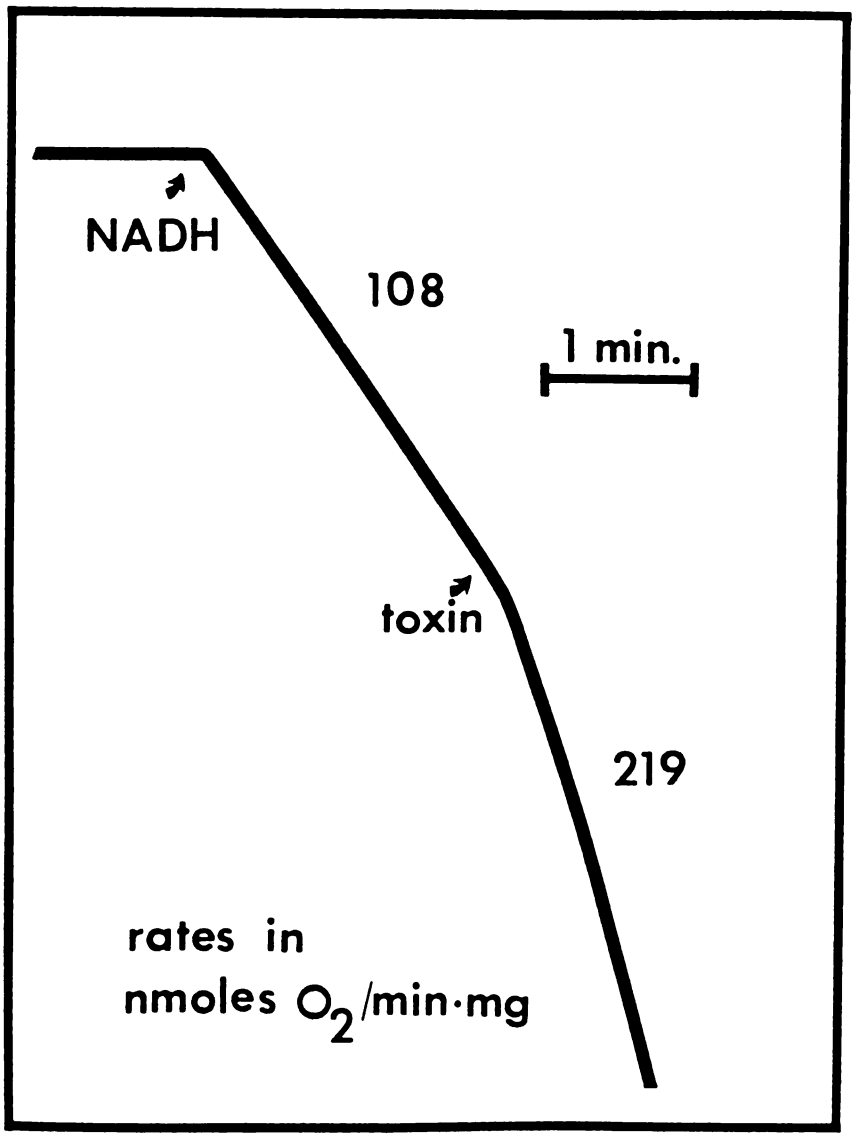

Figure 3. Effects of *Phyllosticta maydis* toxin on NADH oxidation by mitochondria from T-cytoplasm corn. The reaction mixture (3.3 ml final volume) contained KCl (0.2 M), Hepes-NaOH (20 mM, pH 7.5), and $MgCl_2$ (2 mM). Mitochondria in suspension (0.3 ml) were added 2-3 minutes prior to the addition of NADH (0.5 mM in 3.3 ml final volume). The addition of a 20 µl aliquot of *P.maydis* toxin (0.4 µg toxin per ml final volume) is indicated by the second arrow. This particular toxin preparation had an ED_{50} value of 7.2 µg toxin per ml in the CO_2 fixation bioassay. Rates of oxygen consumption are expressed as nmoles O_2/minute·mg mitochondrial protein.

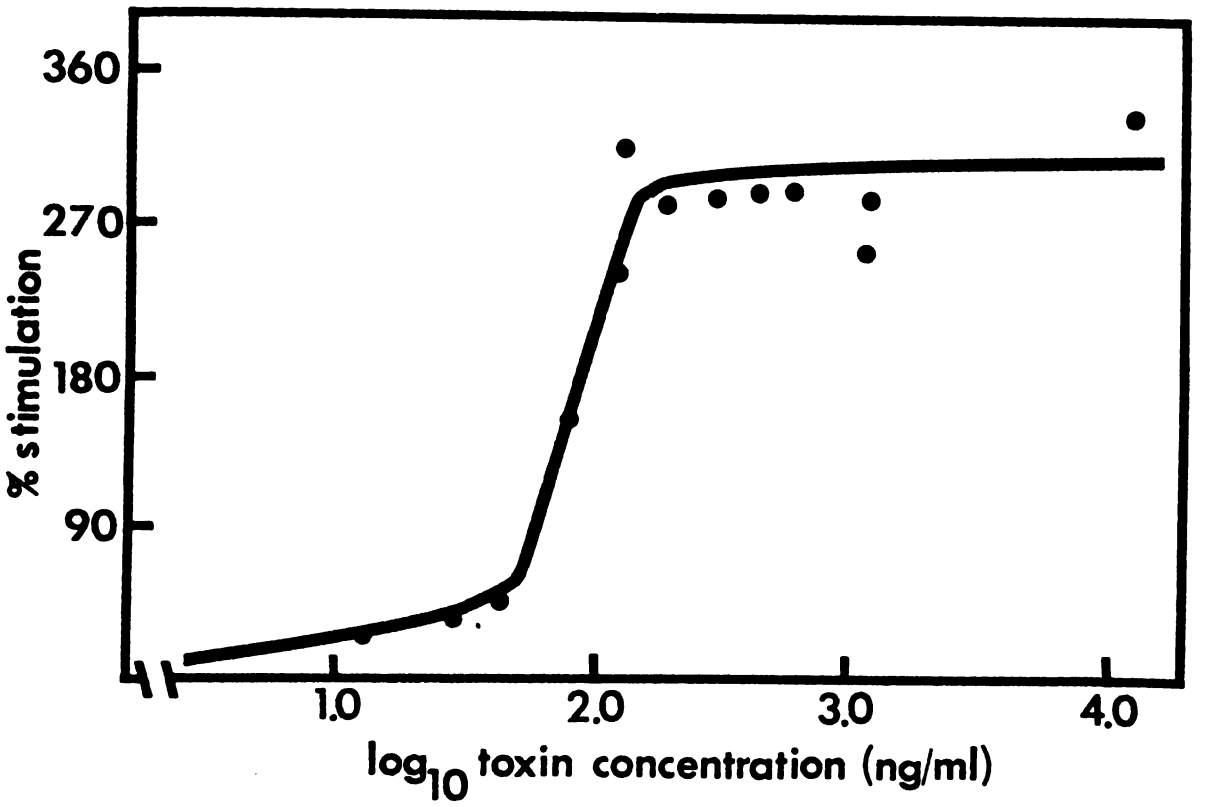

Figure 4. Effects of various concentrations of *Phyllosticta maydis* toxin on the oxidation of NADH by mitochondria from susceptible corn. The reaction mixture is given in Fig. 3. Various concentrations of *P. maydis* toxin were added and their effects on oxygen uptake are given as the per cent stimulation over the original rates for nonphosphorylating mitochondria. This is the same toxin preparation used in the experiment reported in Fig 3.

increase in oxygen consumption rates were recorded. Very high levels of toxin greatly stimulated oxygen consumption while lower levels of toxin caused a less dramatic stimulation. The lag time between the addition of toxin and the increase in oxygen consumption was also affected by toxin concentration. High concentrations of the toxin caused a change in the rate of oxygen consumption in less than 10 seconds, whereas lower levels had a lag time as long as 60 seconds. Mitochondria from insensitive (N-cytoplasm) corn were not affected by 80 µg toxin per ml. The insensitive mitochondria exhibited all the expected responces to ADP and 2,4-dinitrophenol.

Effects of *P.maydis* toxin on oxygen consumption by mitochondria with succinate, malate+pyruvate or NADH as the substrate

Oxygen consumption was also used to measure the effects of *P. maydis* toxin on sensitive mitochondria when succinate or malate+ pyruvate was used as the substrates. Experiments were performed identically to those using NADH as the substrate. Mitochondria from sensitive (T) and insensitive (N-cytoplasm) corn were incubated in the reaction medium which contained Pi but no ADP or substrate. One of the substrates was then added and the nonphosphorylating rate was measured. Next, *P.maydis* toxin was added and the changes in the nonphosphorylating rates were measured. In other experiments, ADP was added after a stable nonphosphorylating rate was obtained. After the resulting phosphorylating rate was obtained, *P.maydis* toxin was added and the resulting changes were noted. Results of a representative experiment are shown in Table 1.

Table I. The effects of *Phyllosticta maydis* toxin on respiration by mitochondria from T-cytoplasm corn, using phosphorylating and non-phosphorylating conditions. Conditions for these experiments are given in Fig. 3. NADH was used at 0.5 mM, malate+pyruvate wer 10 mM each, and succinate was used at 9 mM. To establish phosphoylating conditions, ADP (150 µM) was added 2-3 minutes after the addition of one of the substrates, and 2-3 minutes before the addition of toxin (0.4 µg/ml). Stimulation (+) or inhibition (-) of sensitive mitochondria in response to the addition of toxin was calculated by comparing the toxin-induced rate to the respiration rate immediately preceeding the addition of toxin. This toxin preparation had an ED_{50} value of 7.2 µg/ml in a CO_2 fixation bioassay. Respiration rates are given in nmoles O_2 consumed per minute per mg mitochondrial protein.

Substrate	Nonphosphorylating conditions			Phosphoylating conditions		
	-toxin	+toxin	(%change)	-toxin	+toxin	(%change)
NADH	118	260	(+120)	257	339	(+32)
mal+pyr	25	10	(-61)	66	10	(-85)
succinate	71	40	(-44)	101	67	(-34)

P.maydis toxin stimulated the original nonphosphorylating respiration rate when NADH was used as the substrate. There was also a slight increase in oxygen consumption when toxin was added under phosphorylating conditions. However, the toxin-induced rates under phosphorylating conditions were higher than the toxin-induced rates under nonphosphorylating conditions. The toxin completely abolished respiratory control of sensitive (T-cytoplasm) mitochondria. Following the addition of toxin, ADP no longer affected the respiration rate in sensitive mitochondria. Mitochondria from N-cytoplasm corn retained their respiratory control after toxin treatment.

Using succinate as the substrate, *P.maydis* toxin inhibited oxygen uptake 44% under nonphosphorylating conditions and 34% under phosphorylating conditions. The response of succinate-induced respiration was dose dependent, but very high concentrations of the toxin (65 µg/ml) never completely inhibited oxygen consumption. The response to toxin saturated at about 45% inhibition. With succinate as the substrate, toxin abolished respiratory control in sensitive mitochondria, as it did when NADH was the substrate. Toxin had no effect on mitochondria from N-cytoplasm corn when succinate was the substrate.

The oxidation of malate+pyruvate was inhibited 61% by *P.maydis* toxin under nonphosphorylating conditions, whereas a similar concentration of toxin almost completely abolished oxygen uptake under phosphosrylating conditions. Respiratory control was abolished in sensitive mitochondria. Again, toxin had no effect on mitochondria from N-cytoplasm corn.

DISCUSSION

My experiments were designed to compare the effects of *P.maydis* toxin with the reported effects of the host-selective toxin produced by *Helminthosporium maydis* race T (HmT toxin). Other workers have suggested that the *P.maydis* and HmT toxins may be similar (11, 30). These suggestions were based on the similar genetic control of sensitivity to the toxins and on limited data showing similar physiological responses of sensitive corn to the two toxins.

Sensitivity to HmT toxin is governed by cytoplasmically inherited genes. Corn with Texas male sterile cytoplasm (T-cytoplasm) is sensitive to HmT toxin whereas certain other male sterile types and N-cytoplasm corn is insensitive. Nuclear genes do not appear to have a direct effect on toxin sensitivity. HmT toxin-sensitivity apparently is determined by the T-cytoplasm genes.

The widespread use of T-cytoplasm corn led to the southern corn leaf blight epidemic in 1970. The genetic uniformity of the corn crop caused *H.maydis* to develop into a serious corn pathogen. The toxin gave the special pathogenicity to race T of the fungus. The old races of *H.maydis* remained as weak pathogens. *P.maydis* appears to have exploited the same genetically uniform corn crop. This fungus also developed the ability to produce a host-selective toxin which attacks corn with T-cytoplasm. As with HmT toxin, *P.maydis* toxin increased the pathogenicity of the fungus to corn with T-cytoplasm; all other genotypes appear to be insensitive to the toxin.

These toxins induce specific physiological changes in susceptible seedlings, in green tissues, and in isolated mitochondria.

Some of these effects are similar to those reported for other host-selective toxins, whereas other effects are unique to these two toxins (27).

Many host-selective toxins inhibit root growth in susceptible seedlings (27). HmT toxin also has been shown to inhibit root growth in T-cytoplasm corn seedlings (17). An inhibition of seedling root growth has been reported for *P.maydis* toxin (11,30). I have obtained similar results with a partially-purified preparation of *P. maydis* toxin. This toxin preparation inhibited seedling root growth in T-cytoplasm corn. However, this assay proved unsatisfactory; there was large variation in seedling root growth within each toxin treatment and within the controls. This variability in growth was due in part to the low quality of seed; growth of these imbred seedlings was not uniform enough to limit the amount of variation to an acceptable level.

Many host-selective toxins affect the fixation of CO_2 in the dark. *Helminthosporium carbonum* and *H.victoriae* toxins stimulate CO_2 fixation in plants that are susceptible to the fungus but have no effect in plants that are resistant (27). In contrast, HmT toxin inhibits the fixation of CO_2 by T-cytoplasm corn but has no effect on fixation by T-cytoplasm tissues (9). Inhibition of the dark fixation of CO_2 may be a secondary effect linked to stomatal closure in T-cytoplasm corn. Arntzen *et al.* (3) demonstrated a toxin-induced inhibition of K^+ transport into guard cells; K^+ uptake by guard cells is said to control stomatal action (18).

When T-cytoplasm corn leaf disks were exposed to *P.maydis* toxin, they exhibited lower levels of CO_2 fixation than did leaf disks not exposed to toxin. CO_2 fixation in sensitive tissues was almost completely inhibited by the toxin whereas tissues from N-cytoplasm corn were not affected. The inhibition of CO_2 fixation in T-cytoplasm leaf tissues by *P.maydis* and HmT toxins suggests a similar mode of action. This hypothesis is supported by the fact that other host-selective toxins stimulate rather than inhibit CO_2 fixation in sensitive tissues.

The cytoplasmic inheritance of sensitivity to *P.maydis* and HmT toxins suggests that an altered genome affecting either the mitochondrion or the chloroplast is involved in controlling toxin sensitivity. Many factors seem to eliminate the chloroplasts as a site of action; for example, nongreen tissues and etiolated plant parts are sensitive to the toxin (15). Furthermore, experiments with HmT toxin on chloroplasts isolated from corn have failed to demonstrate toxic effects (2).

Many selective effects of HmT toxin on isolated mitochondria have been demonstrated. These include various changes in the respiration rate with several substrates (22), disruption of the inner mitochondrial membrane (1,25), uncoupling of oxidative phosphorylating from electron transport (7), and swelling (22). Numerous experiments have been designed to demonstrate these effects and to elucidate the exact site and mode of action by HmT toxin.

Although HmT toxin affects T-cytoplasm mitochondria *in vitro*, several workers have questioned the mitochondrion as the primary site of action *in vivo*. Arntzen *et al.* (3) failed to detect effects on T-

cytoplasm mitochondria *in vivo* although this may have been due to technical problems (7). Mertz and Arntzen (21) demonstrated toxin-induced changes in the uptake of inorganic ions, and have suggested the plasma membrane as the primary site of toxin action. Effects on the plasma membrane can be explained on the basis of a toxin/ mitochondrion interaction, since any effects on the respiration of the mitochondria will quickly affect the function of the plasma membrane. Recent ultrastructural experiments with HmT toxin have shown rapid, *in vivo* effects of HmT toxin on mitochondria from T-cytoplasm corn (1). Other experiments have demonstrated rapid changes in the ATP content of toxin treated tissues (29). These results show that the mitochondrion in T-cytoplasm corn contains a direct site of action for HmT toxin.

I have designed several experiments to compare the effects of *P. maydis* and HmT toxins on mitochondria isolated from T-cytoplasm corn. Mitochondrial respiration was followed as oxygen consumption and changes caused by the addition of *P.maydis* toxin were noted. *P.maydis* toxin stimulated NADH respiration when sensitive mitochondria were in a nonphosphorylating condition. When the toxin was added after the addition of ADP, only a small increase in the rates of oxygen consumption was recorded. In both cases, the addition of toxin abolished respiratory control in sensitive mitochondria; additional aliquots of ADP had no effect on respiration. In these and all other experiments, N-cytoplasm mitochondria were not affected by *P.maydis*

toxin at 100-fold higher concentrations. These results confirm and extent the results reported by Comstock *et al.* (11), who reported a stimulation of oxygen consumption in mitochondria form T-cytoplasm corn when NADH was the substrate.

The effects of HmT toxin on mitochondria from T-cytoplasm corn are similar to those of *P.maydis* toxin. Several investigators have reported that HmT stimulated oxygen uptake when NADH was the substrate (7,22). This stimulation was seen under nonphosphorylating conditions. These effects were correlated with the uncoupling of oxidative phosphorylation (29). Respiratory control also was abolished.

P.maydis roxin inhibited mitochondrial respiration when malate+ pyruvate was the substrate under phosphorylating and nonphosphorylating conditions. Again, there was a loss of respiratory control. These results are similar to those previously reported (11). Miller and Koeppe (22) reported that T-cytoplasm mitochondria were also sensitive to HmT toxin when succinate was the substrate. Under phosphorylating conditions, succinate respiration was incompletely inhibited by HmT toxin. The effect was evident with a reaction medium containing KCl; in a sucrose-containing medium, HmT toxin stimulated succinate respiration (24). This difference in the response of sensitive mitochondria is not understood. HmT toxin also uncoupled oxidative phosphorylation and abolished respiratory control in sensitive mitochondria.

It is interesting to compare the substrate-dependent differential effects of the toxins. These different effects may eventually lead to an understanding of the mode of action of these toxins.

The stimulation of NADH respiration is thought to be caused by the uncoupling effects on HmT toxin (15). Many uncoupling agents, such as 2,4-dinitrophenol, stimulate respiration; they allow electron transport to continue but prevent the phosphorylation of ADP to ATP. HmT toxin causes similar effects on the NADH respiration of mitochondria isolated from T-cytoplasm corn. In addition to stimulating NADH respiration, HmT toxin decreases P/O ratios in isolated mitochondria (a measure of uncoupling) (7). An increase in tissue respiration also is seen after HmT toxin treatment of T-cytoplasm corn (8). These effects are all consistent with the action of HmT toxin as an uncoupler.

The inhibition of malate+pyruvate respiration is not well understood and several mechanisms have been reported to explain it (16). A toxin-induced leakage of endogenous NAD from sensitive mitochondria has been observed (16) and could theoretically account for the observed effects of the toxin on mitochondrial respiration. It is possible that toxin-induced swelling *per se* may be the primary mechanism of toxin action (13,16).

P.maydis and HmT toxins appear to have the same site of action. The susceptiblity of T-cytoplasm corn to either toxin implies that the alteration of some cytoplasmically inherited trait may confer the special sensitivity of T-cytoplasm corn to these toxins. The sensitivity of mitochondria from T-cytoplasm corn to toxin suggests that the site of action is within the mitochondrion. The similar responses of T-cytoplasm mitochondria to *P.maydis* and HmT toxins when various substrates were used suggests that the toxins have a similar mode of action. The

exact mode of toxin action has not been determined, but a toxin-induced swelling of sensitive mitochondria may explain the various observed effects.

The question remains: are *P.maydis* and HmT toxins the same chemically? Physiological data suggests that they have the same site and mode of action, but no evidence exists to compare the chemical structures of these two toxins. Although HmT toxin has been purified and a structure suggested (18a), *P.maydis* toxin has not been purified. A final comparison must wait until we have a structure for *P.maydis* toxin.

SUMMARY

Phyllosticta maydis produces a host-selective fungal metabolite: *P.maydis* toxin. Corn with Texas male sterile cytoplasm is susceptible to the pathogen and sensitive to the toxin whereas N-cytoplasm corn is resistant to the fungus and insensitive to the toxin. *P.maydis* toxin inhibited root growth in susceptible seedlings and inhibited CO_2 fixation in the dark in leaf disks isolated from T-cytoplasm corn. Toxin also affected mitochondria isolated from susceptible corn: it stimulated or inhibited respiration (oxygen consumption) depending on the substrate used. Respiratory control was abolished by the addition of toxin to mitochondria from T-cytoplasm corn.

Results obtained with *P.maydis* toxin are similar to those obtained by others with the HmT toxin produced by *Helminthosporium maydis* race T. This suggests that the two toxins may have similar modes of action. The sensitivity of T-cytoplasm corn and mitochondria from T-cytoplasm corn suggests that the two toxins may have similar sites of action as well; T-mitochondria may contain that toxin sensitive site. Taken together, these results suggest that the *P.maydis* and HmT toxins could have similar chemical structures.

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