Plant Physiol. (1980) 66, 477–481
0032-0889/80/66/0477/05/$00.50/0

Effects of Purified *Helminthosporium maydis* Race T Toxin on the Structure and Function of Corn Mitochondria and Protoplasts[1]

Received for publication February 11, 1980 and in revised form April 10, 1980

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ABSTRACT

A toxin preparation from *Helminthosporium maydis* Race T containing several closely related molecules with apparently identical biological activities was highly active against mitochondria and protoplasts from Texas male-sterile (T) cytoplasm corn (T mitochondria and T protoplasts, respectively) but had no effect on their male-fertile (N) cytoplasm counterparts. The toxin preparation caused multiple changes in isolated T mitochondria, including uncoupling of oxidative phosphorylation, stimulation of succinate and NADH respiration, inhibition of malate respiration, increased swelling, loss of matrix density, and unfolding of the inner membrane. Only 6 to 7 nanograms toxin per milligram mitochondrial protein (1.8 nanogram per milliliter) were required to fully uncouple oxidative phosphorylation and to completely inhibit malate respiration in isolated T mitochondria. Similar low concentrations of toxin caused collapse of T protoplasts after several days of culture. Severe ultrastructural damage to mitochondria in T protoplasts was observed within 20 minutes; no changes in other cellular components were observed at this time. These observations on the cytoplasmic specificity, multiple effects, and high activity of the toxin at the mitochondrial and cellular levels highlight its biological significance and potential usefulness in determining the molecular basis of southern corn leaf blight disease.

Cytoplasm specificity in southern corn blight is determined by the interaction between HmT toxin[2] and one or more intracellular sites in T cytoplasm corn. The southern corn blight epiphytotic of 1970, in which a large part of the United States corn crop was destroyed, has led to considerable research into the mechanism of resistance to the disease. Before definitive experiments can be designed, two conditions should be fulfilled. First, the primary site of toxin action at the subcellular level should be known. Second, HmT toxin should be obtained in a relatively pure form.

A growing body of evidence suggests that mitochondria are extremely important, perhaps primary, sites of toxin action. This evidence, which has been reviewed in detail (6, 7), derived from studies in which isolated mitochondria were treated with crude HmT culture filtrate or partially purified HmT toxin. HmT toxin caused several changes in the function and structure of isolated T mitochondria but had no effect on mitochondria isolated from resistant N cytoplasm corn (N mitochondria) (3, 5, 6, 14, 17, 21, 22). Evidence for an effect of the toxin on T mitochondria *in situ* has come from ultrastructural studies in which either roots or mesophyll protoplasts from T and N cytoplasm corn were treated with HmT toxin. Rapid T cytoplasm-specific ultrastructural changes were observed in the mitochondria, but not in any other cellular components (1, 7, 21, 22). Malone *et al.* (12) showed that toxin treatment of T cytoplasm corn leaves resulted in severe mitochondrial damage as indicated by the lack of mitochondrial response to 2-deoxyglucose. Toxin treatment of N cytoplasm corn leaves appears to cause no functional damage to the mitochondria, as indicated by the strong mitochondrial contraction observed on addition of 2-deoxyglucose. Data showing that toxin treatment of T cytoplasm roots leads to a reduction in total and labile phosphates (3) and that toxin treatment of dark-grown T cytoplasm protoplasts (T protoplasts) results in rapid reduction in ATP content (20) also suggest that the toxin affects T mitochondria *in vivo*. No effect on N cytoplasm material occurred in the latter experiments.

There have been several attempts to elucidate HmT toxin structure. Karr *et al.* (8) isolated four toxins, each with a carbon skeleton similar to that of a tetracyclic or pentacyclic triterpenoid. Chemical characterization of these compounds was incomplete and, as reviewed previously (6), their biological significance was unclear. Aranda *et al.* (2) reported that HmT toxin is a derivative of D-mannitol esterified by two acetates and two *N*-formyl-L-valine units. Again, the chemical structure was not fully elucidated and the biological activity of the molecule was not clear. The latest work on HmT toxin structure has been reported by Kono and Daly (10). These authors isolated a highly active and T cytoplasm-specific preparation of HmT toxin. Only 4 to 8 ng toxin were required to kill an entire first true leaf of T cytoplasm corn, whereas 20 μg were applied to N cytoplasm corn leaves without visible effect. The colorless toxin gave an elemental composition of C: 63.84, H: 9.18, O: 26.68, and had a sharp melting point at 125 to 126 C. Several analyses, including spot tests, ^{13}C and proton NMR, IR analysis of the acetylated derivative, and reduction to the hydrocarbon, were interpreted as suggesting a linear molecule of empirical formula $C_{41}H_{68}O_{13}$ with eight carbonyl groups and five free hydroxyls, although other interpretations of toxin structure are possible. Although this toxin moved as a single diffuse component in most chromatographic systems, it gave three major and three to five minor components on commercially prepared EM 60 Silica Gel plates. It was not clear whether these were native components or products of isolation and chromatography. The three main components were nearly identical in structure and biological activity. Although the exact structure of the toxin is not known, its high degree of activity and relative purity make studies of the biological activity of the Kono and Daly (10) toxin preparation important. Work on the finer details of the Kono and Daly (10) toxin structure and comparisons of this toxin with other preparations such as those of Karr *et al.* (8) and Aranda *et al.* (2)

[1] This work was supported in part by a competitive grant from the United States Department of Agriculture, Science and Education Administration, Rockefeller Foundation Grant 75002, and National Science Foundation Grant PCM-7822572. This is Paper No. 701 in the Plant Breeding Series.

[2] Abbreviations: HmT toxin: *Helminthosporium* (*Bipolaris*) *maydis* Race T toxin; T cytoplasm: Texas male-sterile cytoplasm; N cytoplasm: normal, maintainer cytoplasm.

are needed.

We have studied the effects of this toxin preparation (provided by Dr. Daly) on respiration, oxidative phosphorylation, swelling, and ultrastructure of isolated T and N mitochondria. We have also assessed its activity against mesophyll protoplasts from T and N cytoplasm corn by recording changes in survival and ultrastructure of treated protoplasts. The protoplast system was chosen for study of toxin action at the cellular level because it allows rapid and uniform exposure of single cells to HmT toxin and is highly sensitive and T cytoplasm-specific (4).

MATERIALS AND METHODS

Plant Materials. *Zea mays* L. inbred W64A with T or N cytoplasm was used for all experiments. For the mitochondrial experiments, seeds were planted in a fertilized peat-Vermiculite (1:2) mix and grown for 3 days at 30 C in the dark. Plants for the protoplast experiments were grown in the peat-Vermiculite mix under a 12-h photoperiod. After 7 days under a metal halide lamp (27 C), plants were transferred to a growth chamber with cool-white fluorescent lights (50 μE m^{-2} s^{-1}; RH, >90%; 25 C). The youngest leaves on 10- to 17-day-old plants were used for isolation of protoplasts.

Preparation of Mitochondria. Approximately 50 g (fresh weight) of 3-day-old shoots were ground with a mortar and pestle in 100 ml grinding medium consisting of 0.4 M sucrose, 30 mM Hepes (pH 7.4), 50 mM KH_2PO_4, 5 mM EDTA, 1 mM DTT, and 1 mg/ml BSA and strained through four layers of cheesecloth. The strained homogenate was centrifuged at 1,500*g* for 10 min. The supernatant was centrifuged at 12,000*g* for 10 min. The resulting pellet was resuspended with an artist's brush in 100 ml washing medium (*i.e.* grinding medium minus BSA and DTT) and centrifuged at 1,500*g* for 10 min. After centrifugation of the supernatant at 12,000*g* for 10 min the final mitochondrial pellet was resuspended with 1.5 ml medium, which contained 0.4 M sucrose, 20 mM Hepes (pH 7.4), and was kept on ice. All procedures were performed at 0–4 C.

Mitochondrial Assays. Mitochondrial respiration was measured in 3 ml medium composed of 0.4 M sucrose, 20 mM Hepes (pH 7.2), 10 mM KCl, 4 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 1 mg/ml BSA. Respiratory substrates used were 20 mM malate, 10 mM succinate, or 1 mM NADH. Aliquots of ADP at final concentrations of 50 and 100 μM were added in the absence of toxin to condition the mitochondria and to obtain respiratory control ratios, respectively. Toxin was added during the second state 4 condition and a third addition of ADP (100 μM final concentration) was added after an appropriate toxin-treatment time. Measurements of mitochondrial swelling were made under identical conditions to those described for mitochondrial respiration by measuring *A* changes at 520 nm according to the method of Stoner and Hanson (19). Respiratory measurements were made with a Clark-type O_2 electrode (Yellow Springs Instrument Company) and *A* measurements were made with a Cary 219 spectrophotometer. Protein determinations were performed by the method of Lowry *et al.* (11).

Protoplast Isolation and Culture. The techniques used for protoplast preparation were described in detail in Earle *et al.* (4). Leaves were brushed with 320-grit Carborundum, rinsed with distilled H_2O, and floated on an enzyme solution containing 1% Cellulysin (Calbiochem) and 0.5% Driselase (Kyowa Hakko, lot No. K37016) in 0.5 M sorbitol + 10 mM $CaCl_2 \cdot 2H_2O$ (pH 6.0) (sorbitol + Ca). The leaves were incubated at room temperature on a gyrotary shaker (50 rpm) for 3 to 4 h, until they just started to disintegrate. For electron microscopy experiments, enzyme concentrations were doubled; in this more concentrated enzyme solution, incubation time was reduced to 2 to 3 h. After incubation, material was filtered through 80-μm nylon mesh, centrifuged at 100*g*, washed twice in sorbitol + Ca, and resuspended in sorbitol + Ca. Each g leaf yielded 5 to 10 × 10^6 protoplasts.

Protoplasts were cultured in the dark in 35-mm plastic Petri dishes sealed with Parafilm. Each dish contained about 2 × 10^5 protoplasts in a total of 1.0 ml sorbitol + Ca. Protoplasts were treated with toxin by including the desired amount of toxin diluted in sorbitol + Ca. Protoplast populations within sealed plates were examined with a Zeiss-Jena inverted microscope (× 300). Counts of healthy protoplasts were made by examining samples from the culture plates in a Fuchs-Rosenthal hemacytometer with a Bausch and Lomb compound microscope (× 400).

Electron Microscopy. Suspensions of isolated mitochondria were fixed in 1% glutaraldehyde in 0.4 M sucrose, 20 mM Hepes (pH 7.2) (solution A) for 30 min at room temperature (24 C) and then in 3% glutaraldehyde in solution A for 15 min at 4 C. Mitochondria were washed in solution A, postfixed in 1% OsO_4 in solution A for 30 min at 24 C, washed, dehydrated in acetone, and embedded in low viscosity plastic (18) in Beem capsules. Sections were cut with glass knives on a Reichert Om U_2 microtome, poststained with uranyl acetate and lead citrate, and viewed on a Philips EM 300 electron microscope at 60 kv. Protoplast suspensions were fixed in 1% glutaraldehyde in sorbitol + Ca (pH 6.8) for 30 min at room temperature and then in 3% glutaraldehyde in sorbitol + Ca for 15 min at 4 C. Protoplasts were washed in sorbitol + Ca and in 50 mM phosphate buffer, postfixed in 1% OsO_4 in phosphate buffer for 30 min at 24 C, washed, dehydrated in ethanol, and embedded in Spurr's plastic (18) in Beem capsules. Sections were cut with a diamond knife. They were stained and examined as the mitochondrial preparations were.

Preparation of Toxin. The purified toxin preparation was obtained as a gift from Dr. J. M. Daly, University of Nebraska. Isolation and chemical characterization of the toxin was described by Kono and Daly (10). Toxin was prepared for use as follows: 586 μg toxin was dissolved in 58.6 μl warm pyridine. This solution was added to 527 μl methanol to yield 1 mg toxin/ml in pyridine-methanol (1:9). Further dilutions were made in pyridine-methanol (1:10) before treating the isolated mitochondria with toxin. For experiments with protoplasts, the 1 mg/ml toxin stock solution was diluted in sorbitol + Ca (1:1,000 or more) before use.

RESULTS AND DISCUSSION

The toxin preparation, at 319 ng/ml mitochondrial protein, caused several changes in respiration and coupling of isolated T mitochondria but had no effect on N mitochondria, even at 711 ng/mg mitochondrial protein (Fig. 1). Changes in Rc ratios showed that the toxin completely uncoupled the T mitochondria with either malate, succinate, or NADH as respiratory substrates. Respiration with succinate or NADH was stimulated by the toxin but state 4 and state 3 malate respiration was completely inhibited (Fig. 1). These effects of the toxin on T mitochondrial coupling and respiration substantiate data presented in previous reports (7, 15). The ability of this highly purified toxin preparation to cause the observed multiple changes in T mitochondrial respiration and coupling is highly significant. Earlier work with crude HmT culture filtrate or less purified HmT toxin preparations had raised the possibility that multiple toxins are needed to induce these various effects (6, 17). The present results suggest that such multiple toxins are not required, except for the unlikely possibility that the closely related molecules in the toxin preparation were acting on different mitochondrial sites.

The effects of toxin concentration on T mitochondrial activities were assessed by measuring uncoupling of oxidative phosphorylation, with either malate, succinate, or NADH as substrates, and changes in state 4 malate respiration rates. The results (Fig. 2) show that the toxin supplied by Daly is highly active, causing a gradual loss of coupling with all three respiratory substrates in a concentration range of only 0 to 7 ng toxin/mg mitochondrial protein. Complete uncoupling with all three substrates was observed at a concentration of only 6 to 7 ng toxin/mg mitochondrial

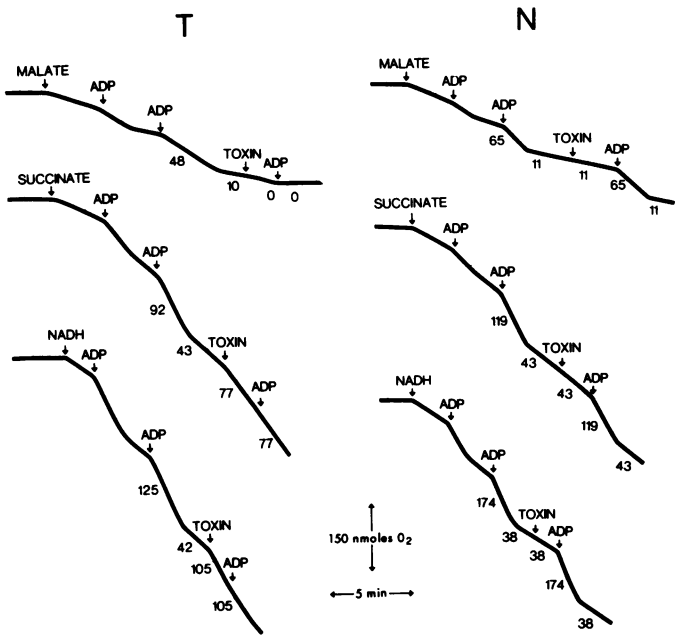

FIG. 1. Effects of toxin on T mitochondrial respiration rates and coupling with malate, succinate, or NADH as respiratory substrates. Where indicated, toxin was added to T and N mitochondria at final concentrations of 319 and 711 ng toxin/mg mitochondrial protein, respectively. The first, second, and third additions of ADP for each trace were at final ADP concentrations of 50 μM, 100 μM, and 100 μM, respectively.

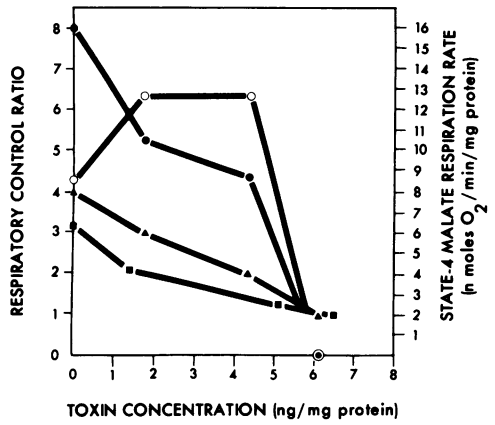

FIG. 2. Effects of toxin concentration on T mitochondrial RC ratio values with malate, succinate, or NADH as respiratory substrates and on state 4 malate respiration rates. ●——●, RC ratio, with malate; ▲——▲, RC ratio, with NADH; ■——■, RC ratio with succinate; ○——○, state-4 malate respiration.

protein (~1.8 ng toxin/ml). State 4 malate respiration was also completely inhibited by 6 to 7 ng/mg mitochondrial protein (Fig. 2). The stimulation of state 4 malate respiration by lower toxin concentrations (up to 4.8 ng/mg mitochondrial protein) was probably due to the rapid uncoupling effect of the toxin at these concentrations (Fig. 2). Payne and Daly (15) and Payne *et al.* (16) also studied the effects of the various concentrations of the same toxin on T mitochondrial respiration and coupling. In contrast to our findings, they found that, at certain concentrations (5 to 20 ng/ml), the toxin uncoupled and stimulated NADH and succinate respiration without significant uncoupling or inhibition of malate respiration (15, 16). On this basis, it was suggested that the toxin might act at two separate sites (15, 16), one of which was specifically related to toxin-induced changes in rates and coupling of malate respiration. Our data (Fig. 2) are consistent with our view (7, 13) that a single site of action could lead to all of the observed effects of the toxin. These differences in data are difficult to understand, particularly as identical toxin preparations were used in each laboratory. One explanation is that our data are the more complete and graphically illustrate the effects of toxin concentration, whereas Payne *et al.* (16) tabulated data based on the effects of only two toxin concentrations.

Light-scattering measurements showed that the toxin caused swelling of T mitochondria but had no effect on swelling of N mitochondria (Fig. 3). Comparison of Figures 2 and 3 shows that the toxin caused complete uncoupling of oxidative phosphorylation and complete inhibition of state 4 malate respiration at concentrations which had a minimal effect on T mitochondrial swelling. This suggests that mitochondrial swelling is a secondary effect of the toxin. The primary effect of the toxin is probably exerted upon the coupling mechanism because toxin-induced inhibition of the RC ratio occurred at even lower concentrations than inhibition of state-4 malate respiration (Fig. 2). Peterson *et al.* (17), using crude toxic filtrate from HmT, also showed that the RC ratio is more sensitive to toxin than is state 4 malate respiration.

The toxin caused marked ultrastructural changes in isolated T mitochondria, whereas N mitochondria were unaffected (Fig. 4). T mitochondria which had not been treated with toxin and N mitochondria in the presence of toxin were highly contracted and contained distinct cristae (Fig. 4, A and C). Toxin-treated T mitochondria appeared swollen with a vesiculated inner membrane and a lightly staining matrix (Fig. 4B).

Recently, Koeppe *et al.* (20) showed that the insecticide methomyl (Lannate 90, wettable powder) adversely affected isolated T mitochondria but had no effect on N mitochondria. The qualitative effects of methomyl on mitochondrial respiration, coupling of oxidative phosphorylation, swelling, and ultrastructure were identical to those of HmT toxin. Justifiably, it was suggested that methomyl with its simple, well defined structure might provide a potential means for characterization of the biochemical differences between T and N mitochondria. A disturbing feature of this work is that methomyl, a carbamate compound, and the toxin structure reported by Kono and Daly (10) appeared unrelated and yet apparently exhibited similar qualitative properties. A comparison of the activities of the Kono and Daly (10) toxin and methomyl showed that these compounds are very different in their specific activities against T mitochondria. Methomyl was active only in the 1 to 3 mM range (9) and was, therefore, many orders of magnitude less active than the toxin supplied by Daly which is active at ~1 nM. This suggests that either methomyl differs from the toxin in its mode of action or that it only weakly exhibits the same action as the toxin. In either case, it appears that the toxin should be a primary, and methomyl a secondary, tool in elucidating differences between T and N mitochondria.

The toxin caused damage to T cytoplasm corn mesophyll protoplasts similar to that seen with a partially purified toxin preparation (4). Healthy protoplasts were spherical and highly vacuo-

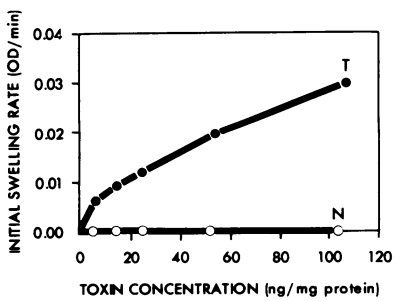

FIG. 3. Effects of toxin concentration on swelling of T and N mitochondria.

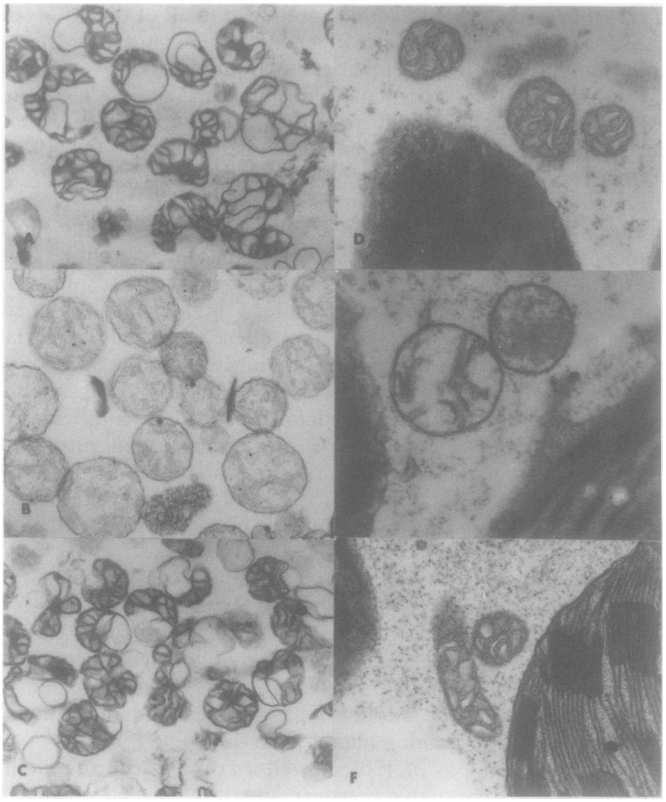

FIG. 4. A–C: isolated corn mitochondria treated with various concentrations of HmT toxin. A, T cytoplasm mitochondria held without toxin for 10 min; B, T cytoplasm mitochondria treated with 85 ng toxin/ml for 10 min; C, N cytoplasm mitochondria treated with 160 ng toxin/ml for 10 min; D–F: mitochondria in corn leaf mesophyll protoplasts cultured in 0.5 M sorbitol + 10 mM $CaCl_2 \cdot 2H_2O$ containing various concentrations of toxin; D, T cytoplasm protoplasts held without toxin (control) for 1 h; E, T cytoplasm protoplasts treated with 500 ng toxin/ml for 20 min; F, N cytoplasm protoplasts treated with 500 ng toxin/ml for 1 h.

lated with distinct disc-like chloroplasts localized at one side (Fig. 5A). Populations of T protoplasts treated with the toxin first showed an increase in protoplasts with indistinct or distended chloroplasts (Fig. 5B). Some of these protoplasts were less vacuolated than untreated controls; others were no longer spherical. Eventually toxin-treated T protoplasts collapsed into dark green cup-shaped clusters of damaged chloroplasts (Fig. 5C).

Table I shows the effects of different toxin concentrations on populations of T protoplasts. The time required for visible damage and collapse of the protoplasts increased as the toxin concentration decreased. With high toxin concentrations (>5 ng/ml), most protoplasts looked abnormal within 24 h and collapsed on the 2nd day after treatment. The lowest active concentration (1.25 ng/ml) caused collapse of all protoplasts after 4 to 5 days. It is striking that the lowest toxin concentrations required for T protoplast collapse and for uncoupling of oxidative phosphorylation and inhibition of state 4 malate respiration were very similar. In different experiments, the timing of the toxin effects at a given concentration varied by 1 to 2 days, but the general pattern was always that shown in Table I. Survival of N protoplasts was not reduced even after treatment with 500 ng toxin/ml for 7 days. These data emphasize the biological significance of the toxin by demonstrating its high activity and cytoplasm specificity at the cellular level.

Although toxin-induced changes in the appearance of T protoplasts were not apparent at the light microscopic level for hours or days, changes in mitochondrial structure were seen by electron

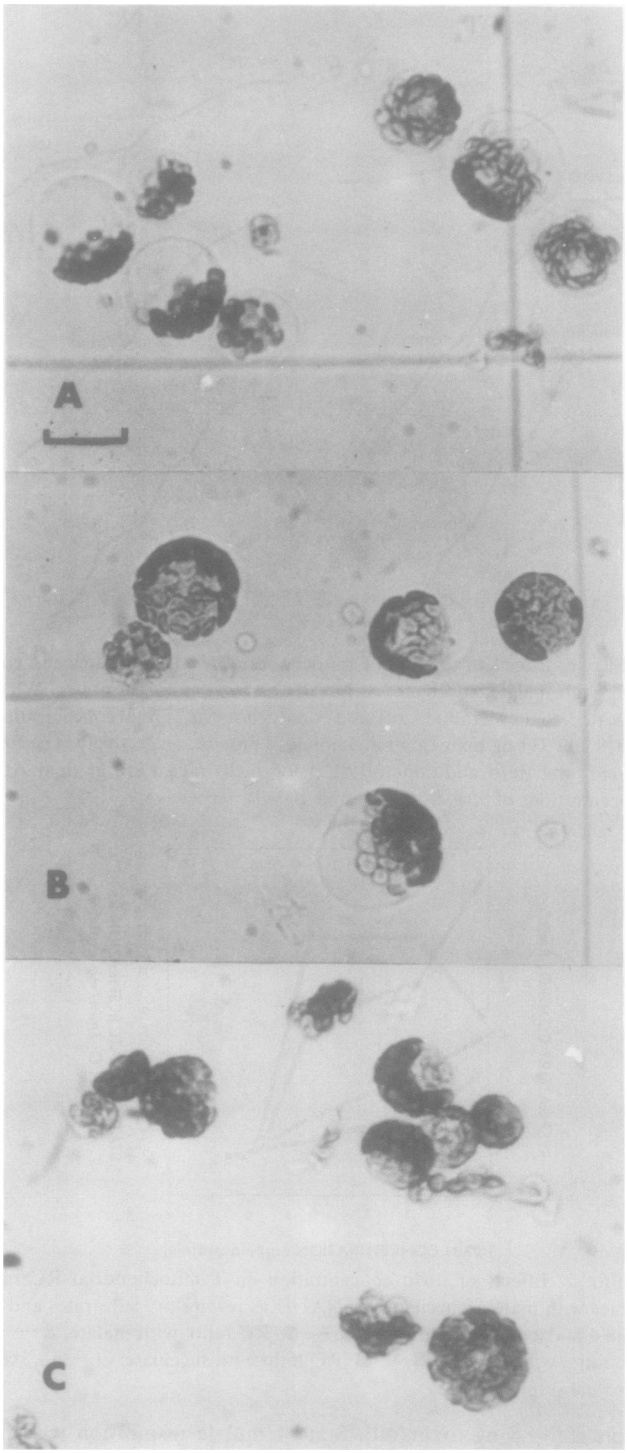

FIG. 5. Leaf mesophyll protoplasts cultured in 0.5 M sorbitol + 10 mM $CaCl_2 \cdot 2H_2O$ containing various concentrations of HmT toxin. The calibration bar represents 25 μm. A, T cytoplasm protoplasts cultured without toxin (control) for 1 day; B, T cytoplasm protoplasts treated with toxin (25 ng/ml) for 1 day; C, T cytoplasm protoplasts treated with toxin (12.5 ng/ml) for 2 days.

microscopy within minutes. Like toxin-treated isolated T mitochondria (Fig. 4B), T mitochondria exposed to toxin *in situ* became swollen and exhibited a loss of matrix density and unfolding of the inner membrane (Fig. 4E). All mitochondria in T protoplasts treated with 500 ng toxin/ml for 20 min showed this type of damage; 15% of the mitochondria treated with 50 ng toxin/ml for

Table I. *Effect of Toxin Concentration and Length of Culture on Survival of T Cytoplasm Corn Mesophyll Protoplasts*

Treatments consisted of 2.6×10^5 protoplasts cultured in 1 ml 0.5 M sorbitol + 10 mM $CaCl_2 \cdot 2H_2O$ containing 0 to 50.0 ng HmT toxin. Healthy protoplasts in the control treatments were spherical and highly vacuolated with distinct disc-like chloroplasts localized at one side. After 1, 2, 3, and 4 days of culture, 60, 53, 53, and 48% of the control protoplasts, respectively, were healthy. The number of healthy protoplasts in each toxin treatment is expressed as a percentage of the healthy control protoplasts on the same day. Each value is the mean of counts of two 3.2-mm^3 samples; this volume of the control treatments contained 400 to 500 healthy protoplasts.

Toxin	Healthy Protoplasts on Following Days after Toxin Treatment			
	Day 1	Day 2	Day 3	Day 4
ng/ml	*% of control*			
0	100	100	100	100
1.25	83[a]	25[a]	3	0[b]
2.5	72[a]	12[a]	0	0[c]
5.0	36[a]	<1[a]	0[b]	0[c]
12.5	1[a]	0[b]	0[c]	0[c]
50.0	0[a]	0[b]	0[c]	0[c]

[a] Many of the unhealthy protoplasts were still turgid, either bulging or spherical with indistinct or distended chloroplasts.

[b] At least 98% of the protoplasts were collapsed. The remaining turgid protoplasts always collapsed within 24 h.

[c] All protoplasts were collapsed. The whole culture plate was examined with an inverted microscope (× 300).

20 min looked abnormal. No other ultrastructural changes in toxin-treated T protoplasts were seen at this time. The T protoplast controls (minus toxin) contained mitochondria with good inner and outer membrane integrity, distinct cristae, and a dark-staining matrix (Fig. 4D). The mitochondria of N protoplasts which had been treated with 500 ng toxin/ml for 60 min were also undamaged (Fig. 4F).

The Daly toxin caused the same multiple T cytoplasm-specific effects on isolated mitochondria and protoplasts as a chloroform-extractable partially purified HmT toxin preparation which had been used in many of our previous experiments (4, 6, 7, 13, 20, 22). The biological significance of the toxin was further emphasized when its activity was compared with that of the chloroform-extractable toxin. The Daly toxin was 20 to 50 times more active than the chloroform-extracted toxin used previously (4, 6, 7, 13, 20, 22) in uncoupling oxidative phosphorylation of T mitochondria and in reducing T protoplast survival. The data suggest that the toxin preparation supplied by Daly is highly active and specific for T cytoplasm. Use of this toxin preparation will facilitate new, better-defined, experiments on the precise molecular mechanisms underlying the mitochondrial and cellular changes effected by HmT toxin in susceptible corn.

Acknowledgments—We would like to thank Leslie A. Kulick, Lydia Potter, and Theresa Wilkinson for excellent technical assistance.

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