

Technique for Using Isolated Corn Root Cap Cells in a Simple, Quantitative Assay for the Pathotoxin Produced by *Helminthosporium maydis* Race T

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ABSTRACT

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Root cap cells from corn with Texas male sterile (Tms) cytoplasm were much more sensitive to HMT-toxin, the pathotoxin produced by *Helminthosporium maydis* race T, than cells from corn with normal (N) cytoplasm. Root cap cells were isolated by gentle agitation of the root in water, and the isolated cells were used to assay HMT-toxin. Each root cap yielded $3-4 \times 10^3$ cells, which were judged to be 98-100% viable by staining with fluorescein diacetate. Toxin-induced cell death was highly temperature-dependent. At 35 C, 5 ng HMT-toxin per milliliter began to

kill Tms cells within 6-8 hr and 50 ng/ml or more caused nearly 100% cell death within 10-12 hr. At least 50 $\mu\text{g/ml}$ was required to kill N-cytoplasm corn root cap cells. The rate of cell death was influenced by cell concentration, but not by plasmolysis. Isolated root cap cells provided a simple, sensitive, quantitative assay for HMT-toxin. The assay was used to compare the lethal effects of HMT-toxin with cell death of oats caused by victorin, the pathotoxin produced by *H. victoriae*.

Additional key words: maize, temperature, *Zea mays*.

Certain plant pathogens produce pathotoxins, compounds that play important causal roles in plant diseases. Pathotoxins are valuable research tools, since they can be used in lieu of pathogens to eliminate many of the complications of working with two actively metabolizing organisms. Selective pathotoxins (20) are especially useful, since they can be used as components in model systems for studying pathogen specificity and the molecular basis for disease resistance.

Recently, increased attention to toxin chemistry has led to the availability of several purified selective pathotoxins (3,14,15). However, lack of standard quantitative assays has hampered efforts to interpret results with toxins from different laboratories, and has limited progress in elucidating pathotoxin modes of action (23). Most assays have employed intact plants or tissues, with consequent problems of uneven penetration and variation in sensitivity of different cell types within a given tissue. Protoplasts can be exposed uniformly, and they respond to toxins (5,8). However, results with toxin-treated protoplasts have been too variable and inconsistent to provide quantitative bioassays (9,23).

Knudson (13) reported that detached root cap cells can survive for weeks in a simple nutrient medium. An assay for victorin, the pathotoxin produced by *H. victoriae*, was developed using isolated oat root cap cells maintained in water (9,10). The assay, based on victorin-induced cell death, was rapid, quantitative, and very simple. It was used to demonstrate that victorin-induced cell death is temperature-dependent and is inhibited by plasmolysis (10). Root cap cells were compared with root cap protoplasts to show that the presence of the cell wall does not influence the lethal effects of victorin. In addition, root cap cells were compared with other cell types to show that cells from different tissues of susceptible plants can vary significantly in sensitivity to victorin (9).

HMT-toxin, the pathotoxin produced by *H. maydis*, race T, has been studied more extensively than any other pathotoxin except victorin. The activity of this compound has been measured by at least 40 different methods (7). The four most commonly used assays

have been evaluated several times (18,21,23,24); none combines simplicity with sensitivity, quantitateness, and speed. Corn root cap cells from lines with the Texas male sterility (Tms) factor are much more sensitive to HMT-toxin than are those of lines with normal (N) cytoplasm (1). The purpose of this study was to adapt the root cap cell assay for use with HMT-toxin and corn and to use the assay to determine the influence of temperature, plasmolysis, and cell concentration on the lethal effects of HMT-toxin. The results were compared with data from a similar study of the effect of victorin on oat root cap cells.

MATERIALS AND METHODS

HMT-toxin. Purified HMT-toxin was a gift from J. M. Daly (Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln). Stock solutions of 10 mg/ml or 10 $\mu\text{g/ml}$ in dimethylsulfoxide (DMSO) were stored at room temperature. Toxin was diluted from DMSO into water, and an equal amount of DMSO was added to control preparations.

Corn seedlings. All studies were conducted using single lots of W64A Tms and W64A N-cytoplasm seeds, which were a gift from Vernon Gracen (Department of Plant Breeding and Biochemistry, Cornell University, Ithaca, NY). To minimize microbial contamination, aseptic protocol was used at all stages of germination. Seeds were soaked for 10 min in 0.05% sodium hypochlorite, rinsed thoroughly, and soaked for 1 hr in water. They were then placed embryo downward between sheets of moistened germination paper in 90-mm-diameter petri dishes, and were incubated at 25-27 C for 36-40 hr, or until the roots were 8-10 mm in length.

Isolation of cells. Root cap cells were harvested by a modification of the procedure used to isolate oat root cap cells (10). Eight to 10 holes (5 mm in diameter) were punched in a vinyl mesh screen. The screen was placed over a petri dish and secured with a rubber band. Seedlings were placed, roots down, through the holes and enough water was added to the dish so that 2-3 mm of the root tips was submerged. After 5-10 min, the dish was agitated manually in a gentle circular motion for approximately 1 min. The cells released by this process were transferred to a beaker, and the cells were agitated vigorously for 5-10 min using a stream of air introduced through a hypodermic needle. The cells were then

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filtered through a 70- μ m-mesh screen, centrifuged at 90 g, and washed four times in water. The concentration was adjusted on the basis of hemacytometer counts to approximately 10^3 cells per milliliter for all studies except the cell concentration study. The cells were then centrifuged again and resuspended in Pfeffer's medium (17) plus 0.01% penicillin G.

One-milliliter samples of cells in capped 15-ml glass tubes were used in the assay. Cell samples were incubated at constant temperatures, and at intervals, 0.1-ml fractions containing approximately 100 cells were examined microscopically. Cell viability was assessed by the ability to stain with fluorescein diacetate (FDA) (10,22).

RESULTS

Cell isolation and viability. Each root cap yielded approximately $3-4 \times 10^3$ cells. Filtering through a 70- μ m-mesh screen removed large clumps of cells, but 20–50% of the cells were in chains or clumps of 2 to 10 cells. Filtering through a 30- μ m-mesh screen resulted in a population of single cells, but significantly reduced the yield and did not influence sensitivity to HMT-toxin. Both individual cells and clumps were included in viability tests.

The population of freshly isolated cells from 36-hr seedlings of W64A Tms and N-cytoplasm cultivars contained 98–100% viable cells. Repeated centrifugation did not reduce viability. Detached cells remained 96–98% viable for at least 12 hr after isolation (Table 1). However, cells incubated in water alone began to die between 12 and 25 hr. Survival was enhanced by incubation in Pfeffer's medium with penicillin. Cell death in the absence of toxin exhibited a temperature coefficient (Q_{10}) of approximately 2.

HMT-toxin effects on isolated root cap cells. *Temperature.* At concentrations from $5-20 \times 10^3$ ng/ml, HMT-toxin killed root cap cells from Tms, but not those from N-cytoplasm corn. Since cells in the controls began to die within 24 hr (Table 1), results of long-term assays (more than 12 hr) were expressed as percentage of living control cells. Toxin-induced Tms-cytoplasm corn root cap cell death was highly temperature-dependent (Fig. 1). At 35 and 37 C, 50 ng or more per milliliter killed most cells within 10–12 hr (Fig. 2). Since no cells in the controls died within this period at these temperatures, all other assays were done at 35 or 37 C.

Cell concentration. Toxin-induced death was influenced by the concentration of cells used in the assay (Table 2), especially at toxin concentrations below 100 ng/ml. For a given cell concentration \pm 200 to 300 cells, the rate varied little among tests. But an increase of 10^3 or more cells resulted in a significant decrease in the death rate. The survival of control cells was not influenced by cell concentration, or DMSO.

Dosage response. At 35 C, toxin at 5 ng/ml caused a significant increase in death of Tms-cytoplasm cells within 6–8 hr (Fig. 2). Toxin concentrations at 50 ng/ml or greater caused death of nearly all cells within 12 hr. Apparently saturation occurred at concentrations of ~ 500 ng/ml; higher toxin concentrations caused no significant increase in the rate of cell death.

Fifty and 100 μ g of HMT-toxin per milliliter caused significant death of N-cytoplasm cells, but viability in 20 μ g/ml did not differ

TABLE 1. Effect of temperature and time on viability of isolated Tms corn root cap cells^a

Time (hr)	Percentage of cells viable at the following temperatures (C) ^b			
	20	25	30	35
6	96 \pm 1	96 \pm 1	98 \pm 1	98 \pm 4
12	96 \pm 2	98 \pm 2	98 \pm 3	97 \pm 3
25	92 \pm 5	90 \pm 4	72 \pm 6	50 \pm 3
30	87 \pm 7	75 \pm 6	52 \pm 8	0
48	81 \pm 7	62 \pm 3	0	0

^aCells were incubated in Pfeffer's medium plus 0.01% penicillin. Viability was assessed by the ability to stain with FDA.

^bValues are means and standard errors from a total of 200–300 cells in at least three experiments.

from that of controls (Table 3). At 35 C, cell death at 50 μ g/ml did not differ significantly from controls for 10 hr. Although toxin preparations were cloudy at 50 and 100 μ g/ml, cell death was more rapid at 100 μ g/ml. At this concentration, cells were surrounded by clumps of material that appeared to be undissolved toxin. Toxin-induced death of resistant cells was also influenced by temperature; for at least 12 hr, viability of N-cytoplasm cells incubated at 25 C, even in 100 μ g of toxin per milliliter, did not differ from controls.

Plasmolysis. Plasmolysis had no effect on HMT-toxin-induced death of Tms cells. Cells that were plasmolyzed for 20 min in 0.6 M sorbitol prior to toxin exposure died at the same rate as nonplasmolyzed cells (Table 4).

TABLE 2. Effect of Tms cytoplasm corn root cap cell concentration^a on cell death induced by HMT-toxin

Concentration of (cells per ml)	Percentage of cells viable after:	
	5 hr	6 hr
HMT-toxin		
1,000	24 \pm 2	16 \pm 4
2,500	32 \pm 5	28 \pm 5
5,000	65 \pm 3	43 \pm 5
10,000	72 \pm 3	52 \pm 6
20,000	78 \pm 6	70 \pm 8
Control		
1,000	97 \pm 1	97 \pm 1
20,000	96 \pm 2	97 \pm 1

^aCells were incubated at 37 C in Pfeffer's medium with penicillin, with 50 ng HMT-toxin per milliliter. Viability was assessed by the ability of cells to stain with FDA. Each value represents the mean and standard error from a total of 200–300 cells in at least three experiments. Cell concentrations were adjusted using a hemacytometer. Control was treated similarly but did not receive toxin.

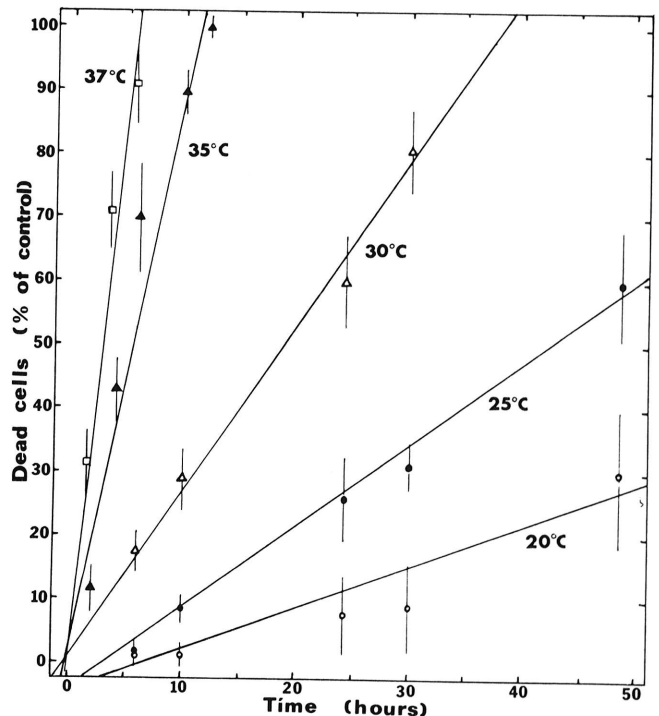


Fig. 1. Effect of temperature on HMT-toxin induced Tms cytoplasm corn root cap cell death. Cells were treated with 100 ng/ml HMT-toxin and the number killed was calculated as the proportion of the live cells in a non-toxin-treated control maintained under the same conditions. Values represent means from a total of 300–500 cells in three experiments. Vertical bars indicate standard errors of the means. Best-fit lines were calculated by linear regression analysis of mean values; correlation coefficients (r) for 20–37 C were, respectively, 0.915, 0.99, 0.998, 0.98, and 0.99.

TABLE 3. Effect of HMT-toxin on viability of root cap cells^a from W64A N-cytoplasm corn

Time (hr)	Percentage of viable cells at the following concentrations of HMT-toxin:			
	Control	20 µg/ml	50 µg/ml	100 µg/ml
4	98 ± 1	98 ± 0	98 ± 1	98 ± 1
6	97 ± 2	96 ± 4	95 ± 1	81 ± 4
8	95 ± 3	92 ± 4	86 ± 2	52 ± 1
10	98 ± 1	92 ± 5	92 ± 1	34 ± 2
12	98 ± 1	94 ± 1	88 ± 1	22 ± 2
25	67 ± 3	71 ± 7	34 ± 2	0

^a Cells were incubated at 37 C in Pfeffer's medium plus 0.01% penicillin G, with HMT-toxin. Viability was assessed by the ability to stain with FDA. Each value represents mean and standard error from a total of 150–300 cells in at least two experiments. Control was treated similarly but did not receive toxin.

TABLE 4. Effect of HMT-toxin on viability of plasmolyzed and nonplasmolyzed Tms corn root cap cells

Time (hr)	Percentage of cells alive ^a			
	Control		HMT-toxin	
	Nonplasmolyzed	Plasmolyzed	Nonplasmolyzed	Plasmolyzed
2	95 ± 2	94 ± 3	80 ± 3	79 ± 5
4	94 ± 4	96 ± 2	67 ± 6	70 ± 2
6	94 ± 3	92 ± 4	54 ± 3	57 ± 5
8	96 ± 1	90 ± 8	30 ± 3	31 ± 4

^a Cells were suspended in Pfeffer's medium with penicillin at a concentration of 10³ cells per milliliter. The suspension was divided into two samples and cells in one sample were plasmolyzed by the addition of 0.6 M sorbitol. After 20 min, 100 ng HMT-toxin per milliliter was added to 1-ml cell samples, and the cells were incubated at 35 C. Cell viability was assessed by the ability to stain with FDA. Each value represents the mean and standard error from a total of 150–200 cells from two experiments.

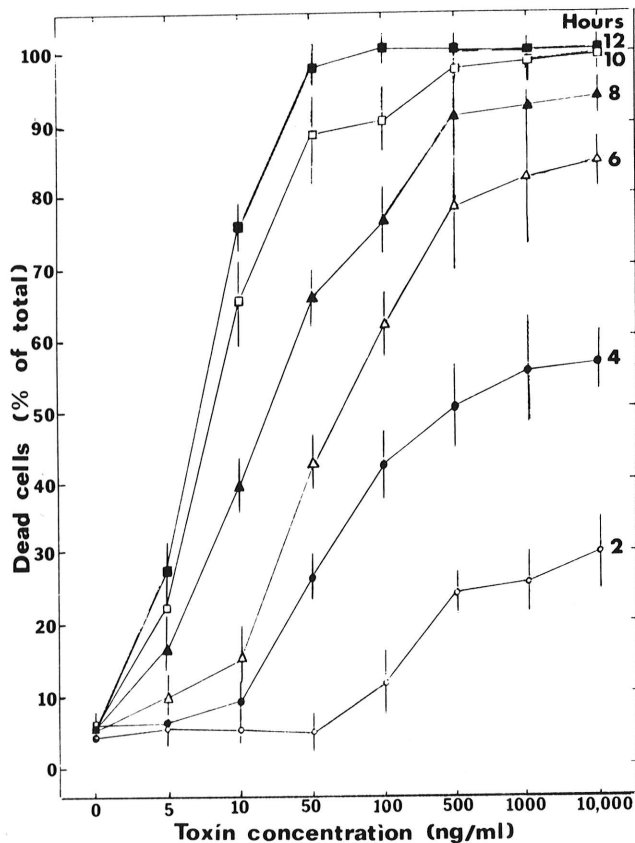


Fig. 2. Effect of toxin concentration on the rate of HMT-toxin induced Tms cytoplasm corn root cap cell death. Cells were incubated at 35 C in Pfeffer's medium with 0.01% penicillin in the indicated toxin concentrations. Points are means from a total of 350–600 cells in at least three experiments; vertical bars represent standard errors of the means.

DISCUSSION

Detached corn root cap cells can survive for weeks in Pfeffer's medium in the presence of intact roots (13). However, unlike isolated oat root cap cells, which survived for up to 1 mo in water alone (10), isolated corn cells began to die in water within 1 day after being harvested. A nutrient medium without hormones significantly enhanced survival of the detached cells, and it is possible that roots incubated with the cells (13) supplied some other nutrient necessary for long-term survival. In any case, at high temperatures, HMT-toxin killed all susceptible cells before control cells began to die, so that survival in Pfeffer's medium was satisfactory for the purposes of the toxin assay.

Tms-cytoplasm cells were at least 10⁴-fold more sensitive to toxin

than were N-cytoplasm cells. The fact that toxin concentrations greater than 500 ng/ml did not significantly increase the rate of death of Tms cells suggests that available sites of action for lethal effects became saturated. The decrease in death rate at high cell concentrations is presumably due to reduction in available toxin due to binding to the cells. Although the data do not establish whether the effect involves specific binding to sites of toxin action, or nonspecific interactions, the results indicate that population size should be considered when comparing pathotoxin studies that employ isolated cells or protoplasts.

The enhancement of the lethal effects of HMT-toxin by high temperatures was not surprising in view of the fact that many of the processes affected by HMT-toxin, such as respiration, transpiration, and electrolyte leakage (4), are also temperature-dependent. In the past, assays have been conducted at 22, 24–28, 30, and 38 C (1,2,6,8,11,12,16,19). Since an increase of only 2 C caused a significant increase in cell death rate, it is apparent that use of different temperatures could lead to much variability in results, and it is thus very important to carefully control temperature regimes when studying the effects of HMT-toxin.

The lack of effect of plasmolysis on HMT-toxin-induced cell death indicates that effects of HMT-toxin on corn cells can be compared with reported effects (7,8) on isolated protoplasts. The results of the current study suggest that corn root cap cells are less sensitive than mesophyll protoplasts to HMT-toxin. Mesophyll protoplasts from Tms-cytoplasm corn, which were incubated at room temperature, were all killed after 1 day in 50 ng purified HMT-toxin per milliliter (8), while 50% of root cap cells incubated at 25 C remained alive after 2 days at the same concentration of toxin. Gregory et al (7) also found that mesophyll cells are more susceptible to ultrastructural damage by HMT-toxin than root cap cells.

The use of a single quantitative assay for both victorin and HMT-toxin allows the direct comparison of factors that influence pathotoxin induced cell death. For example, the lethal effects of both toxins were highly temperature-dependent and, in both cases, mesophyll cells of susceptible plants were much more toxin-sensitive than were root cap cells (9). However, plasmolysis, which strongly inhibited victorin-induced cell death (10), did not influence the effects of HMT-toxin. The elimination of many variables inherent in using different assays can facilitate comparison and interpretation of toxin effects, and may contribute to a better general understanding of pathotoxin roles in plant disease. Preliminary results indicate that corn, sorghum, and sugarcane cultivars susceptible and resistant to *H. carbonum*, *Periconia circinata*, and *H. sacchari*, respectively, also have viable root cap cells that can be harvested and maintained in water. Thus, it may be possible to develop the root cap cell assay for use with the pathotoxins produced by these pathogens as well as those that produce victorin and HMT-toxin.

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