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Inhibition of Dark CO_2 Fixation and Photosynthesis in Leaf Discs of Corn Susceptible to the Host-specific Toxin Produced by *Helminthosporium maydis*, Race T[1]

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ABSTRACT

The host-specific toxin produced by *Helminthosporium maydis*, race T, causes 50% inhibition of dark fixation of $^{14}CO_2$ by leaf discs of susceptible (Texas male sterile) corn when it is diluted to approximately 1/10,000 of the volume of the original fungus culture filtrate. Dilutions of 1/10 or less are required for equivalent inhibition of discs prepared from resistant (N) corn. Root growth and photosynthesis were considerably less sensitive (dilution values 1/3000 and 1/1200, respectively), as was leakage of ^{14}C induced by toxin from preloaded discs. Based on literature values for dilutions causing ion leakage or inhibition of mitochondrial oxidation, toxin dilutions several orders of magnitude greater bring about inhibition of dark CO_2 fixation. Preincubation of discs in light increased sensitivity of dark fixation to toxin and an effect of light on symptom development was shown. Phosphoenolypruvate carboxylase activity in extracts of roots or leaves was not affected by toxin nor was the enzyme level altered in excised leaves treated with toxin. Inhibition of dark fixation of CO_2 provides a bioassaay for race T toxin which is both reliable and rapid.

The host-specific toxin produced by *Helminthosporium maydis*, race T (12), affects a variety of processes in corn cultivars possessing Texas male sterile (Tms) cytoplasm. It had been suggested (13), but later modified (2), that the primary effect may lie in a disturbance of mitochondrial oxidation. A mitochondrial target site would be consistent with increased leakage of ions (6, 7) and inhibition of root growth and pollen tube elongation (11) of corn susceptible to the toxin. In addition to these effects, a drop in transpiration and photosynthetic rates caused by toxin was ascribed to stomatal closure induced by alteration of K^+ flux in guard and subsidiary cells (1). At the enzyme level, there appears to be specific inhibition by the toxin of an ATPase present in the microsomal fraction obtained only from Tms corn (28).

Host-specific toxins affecting oats (3, 17), corn (18, 20), and sorghum (20) show similar multiple effects. The leakage of ions induced by most of these toxins has centered attention on host membranes as a site of action (5, 19, 21, 22). A major problem in the identification of a primary site for race T toxin is the considerable variation in toxin dosage and time of exposure that has been reported for each effect. In this laboratory, for example, 50% inhibition of susceptible corn root growth can be achieved with 1/3000 dilution of race T toxin, whereas dilutions of 1/10 to 1/100 apparently are required for rapid induction of ion leakage (6, 7) or inhibition of mitochondrial oxidation (9, 13, 16).

At present, it is impossible to decide whether the variations arise from differences in toxin potency, the nature of the assay systems employed, or fundamental differences in the innate sensitivity of the processes under examination. It seems reasonable that the primary site would show the greatest sensitivity; that is, would require the least amount of toxin for a response.

As a first approach to the problem, a quantitative bioassay for standardizing activity of toxin from various sources becomes essential. Inhibition of root growth has been used for other host specific toxins. Although usable for race T toxin, we have found this method time-consuming, tedious, and unreliable because of variations in seed germination and seedling vigor. During studies of the effect of T toxin on several physiological and biochemical processes of corn leaves, we observed significant reduction in dark fixation of CO_2. In addition to its intrinsic interest in connection with a mode of action, we also have found the phenomenon to be useful as a reliable, sensitive, and rapid bioassay.

MATERIALS AND METHODS

Corn inbred lines W64A (N-cytoplasm) and W64A-Tms (T-cytoplasm) were grown in a controlled environment at 26 C under a 14-hr photoperiod of fluorescent light at 2500 ft-c.

Toxin was partially purified by extracting crude culture filtrates and aqueous homogenates of ground mycelium with 4 volumes of chloroform. The chloroform from the combined solutions was removed under vacuum, and the residue was dissolved in a volume of H_2O equal to the original volume of modified Fries' medium (18) upon which *Helminthosporium maydis*, race T, was grown. For bioassay, the toxin was diluted in 10 mM KH_2PO_4 and all values are reported as dilutions of the original volume of culture medium.

Dark CO_2 Fixation. Discs (5 mm diameter) from true leaf No. 4 of 19- to 21-day-old plants were cut with a No. 2 cork borer (5 mm diameter) and floated on water. Leaves 1 through 3, although responding to toxin similarly, had less vascular tissue and were difficult to cut without excessive damage. Discs (10–12) were randomly transferred to steel planchets (25 ×

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7 mm), usually 22 to 24 per assay with each containing 2.8 ml of solution, in a 150 × 25 mm plastic Petri dish modified to serve as a "$^{14}CO_2$ tagging" chamber. The leaf discs were preincubated, in the presence of toxin, under 1300 ft-c of fluorescent light for 6 hr at 26 C before permitting fixation of $^{14}CO_2$ in the dark.

A planchet containing 1 ml of 3 N H_2SO_4 then was placed in the center of the chamber. After sealing with vacuum grease, the chamber was covered with aluminum foil, and $^{14}CO_2$ was released by injecting 0.5 μmole of $NaH^{14}CO_3$ into the acid through a serum stopper. After correcting for the volumes of planchets and liquid, the amount of CO_2 in the air space of the chamber was 3.5 μmoles (specific radioactivity approximately 1×10^7 dpm/μmole). The air inside the chamber was mixed quickly with the help of a large syringe.

After 2 hr of exposure to $^{14}CO_2$ in the dark, the chamber cover was removed in a fume hood. After allowing the discs to sit for 15 min in the dark, 4 to 10 discs were transferred to a scintillation counting vial containing 0.5 ml of digestion mixture, prepared by mixing 60% perchloric acid and 30% H_2O_2 in 1:2 ratio. The vials were capped and incubated for 1.5 hr at 70 C. After cooling, 10 ml of scintillation fluid were added. Scintillation fluid was prepared by mixing 15 g of Omnifluor (New England Nuclear), 1 liter of toluene, and 1 liter of Triton X-100. Samples were counted in a Nuclear-Chicago liquid scintillation spectrometer.

In carrying out dark CO_2 fixation assays, it was observed occasionally that fixed CO_2 was released in the first few minutes after discs were collected. To minimize these effects, we routinely removed all replicates of a dilution series in planned sequence rather than at random. In addition, nontoxin-treated control discs were harvested at the start, near the middle, and at the end of a dilution series.

Photosynthetic $^{14}CO_2$ Uptake. After preincubation of leaf discs as above, no more than 300 discs in planchets were transferred to Plexiglas chambers of 1.6 liters held at 26 C in the growth rooms in which corn was grown. While the chambers were darkened, sufficient $^{14}CO_2$ was generated to provide 0.1% in the gas phase. After 10 to 15 min, the discs were exposed to fluorescent light at 2500 ft-c. In tests with 200 nontreated discs per chamber, the gas phase was sampled periodically. After a lag of 3 to 4 min in the light, uptake was linear for at least 40 min. The CO_2 concentration was reduced by half at 20 to 25 min. Consequently, additional $^{14}CO_2$ was generated at that time during experiments in order to restore the original concentration. Photosynthetic $^{14}CO_2$ uptake was terminated by darkening at 50 min and processing the discs as for dark CO_2 fixation.

Phosphoenolpyruvate Carboxylase (EC 4.1.1.31) Assays. Extracts of leaves and washed roots of corn were prepared essentially as described by Ting and Osmond (27). PEP[2] carboxylase activity was measured by NADH oxidation in the presence of 1000 added units of malate dehydrogenase (27). Extract equivalent to 0.1 g of root tissue yielded suitable reaction rates, while amounts equivalent only to 0.02 to 0.04 g were satisfactory for leaf material.

Root Bioassay. Our best results were obtained with the following procedures. Corn seeds, water-soaked for 8 to 10 hr, were surface-sterilized with 70% alcohol for 1 min and placed on sheets of moistened germination paper, which then were rolled into a cylinder. After covering with aluminum foil, they were incubated at 26 C for approximately 40 hr. Next, seedlings selected for root lengths of 5 mm were placed between two unrolled freshly moistened papers. After approximately 8 additional hr, roots with a uniform length of approximately 10 mm were selected and marked with a black felt pen about 5 mm from the root tip. Twenty-five ml of appropriate dilutions of toxin were placed in sterilized Petri plates containing Whatman No. 1 filter paper along with five T and five N seedlings. Each dilution was run in triplicate. Roots were measured after 48 hr of incubation in a dark chamber at 26 C. Root growth was measured from the black mark to the tip, and 5 mm were substracted for the final value of root extension in mm.

RESULTS

With host-specific toxins of high potency and/or hosts of great sensitivity, such as victorin and oats in which effects can be noted at dilutions of 10^6 or 10^7, the dilution causing 50% inhibition can be estimated satisfactorily from graphs. With race T toxin, the range of dilutions for which effects can be measured quantitatively is not as large and statistical treatment was found to be important. A program was written utilizing the statistical hardware of a Monroe 1860 calculator in which individual measurements, or averages constituting replicates, were converted to percentage of control (no toxin treatment) at each dilution value, expressed as the negative log of dilution (P dilution). When ^{14}C was a component of the bioassay, a program subroutine converted gross counts to dpm.

The calculator programs generated the regression line, correlation coefficient, the dilution, or concentration at 0% of control (intercept with x-axis) and at 50% of control. The literature on toxin is concerned with dilution values for percentage inhibition of a process (100 minus per cent of control) but when percentage inhibition is plotted, the intercepts of either the x- or y-axis have no physical significance. For consistency with previous literature, the data were converted to percentage inhibition. For comparative purposes, the dilution corresponding to 50% inhibition was preferred because it is least influenced by sample variation, found particularly at higher dilutions.

Dark Fixation of CO_2. Figure 1 shows the effect of race T toxin, preparation A, on the dark fixation of CO_2 by W64A-Tms and W64A-N corn leaves. The points of Figure 1 consist of the average of three replicates but the regression line was generated from individual values of the replicates.

The fixation by control, nontreated tissue amounted to approximately 0.045 mg CO_2 dm^{-2} hr^{-1}, a typical value under these conditions.

The protocol in these studies included a 6-hr exposure to light prior to the period of dark fixation of CO_2 for several reasons. Previous experience with wheat leaves (4) had suggested a diurnal increase in dark CO_2 fixation rates caused by light. Other studies indicated an effect of light on the action of toxin in corn leaves (see "Discussion"). Table I indicates that the length of the light pretreatment determines sensitivity of dark fixation to the toxin.

The data of Table I do not permit a distinction to be drawn between effects of light and effects caused by length of exposure to toxin, but the experiment summarized in Figure 2 shows that light has an influence on toxic action. All discs were placed in buffer and toxin at the indicated dilutions for 0.5 hr under 1300 ft-c for fluorescent light, rinsed with distilled H_2O, then transferred to buffer alone for 6.5 hr. Dark fixation was carried out for 1 hr in 0.1% CO_2. The shortened time of exposure to toxin decreased the dilution causing 50% inhibition from approximately 10,000 (Fig. 1) to 3000 at the usual light

[2] Abbreviations: PEP, phosphoenolpyruvic acid; HPMS: 2-pyridyl hydroxymethane sulfonic acid.

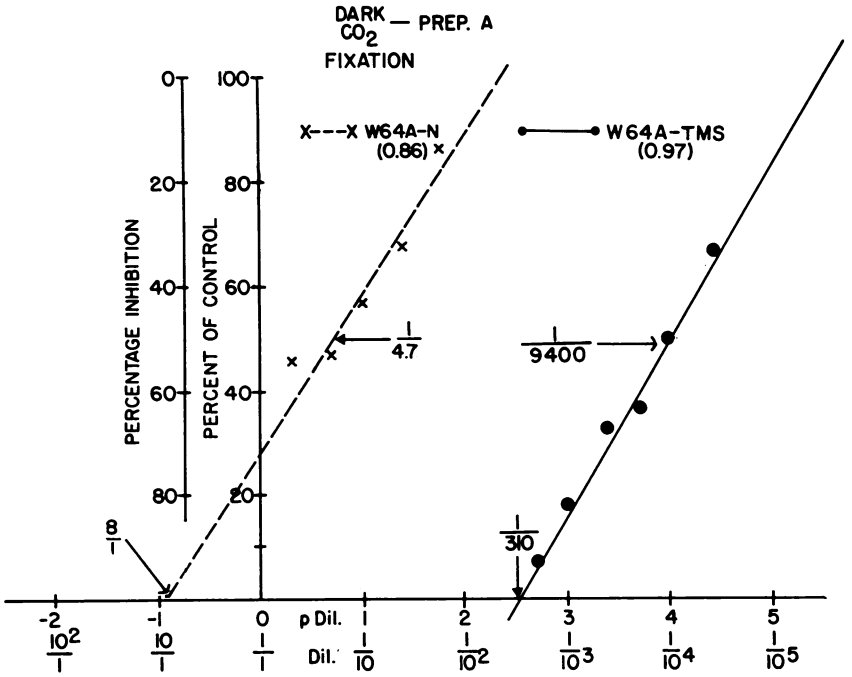

FIG. 1. Inhibition of dark CO_2 fixation of corn leaf discs by race T toxin, preparation A. N corn, resistant (×); Tms corn, susceptible (●). The number in parenthesis is the correlation coefficient of the best-fit line generated by individual replicates. Arrows pointing toward abscissa indicate the dilutions required for 100% inhibition. Arrows pointing toward the ordinate indicate dilution for 50% inhibition.

intensity of 1300 ft-c for the bioassay. At each dilution, however, the inhibition observed at a light intensity of 300 ft-c was significantly lower, resulting in a calculated dilution of 1/600 for 50% inhibition of fixation.

The concentration of CO_2 and the length of time for dark fixation also was examined. Total fixation increased with time at 0.03% CO_2 (15,000, 22,900, and 27,600 dpm/untreated disc at 1, 2, and 3 hr, respectively), but the percentage of inhibition was not affected appreciably. One hr fixation of 0.1% CO_2 was the equivalent of 2 hr at 0.03%.

Arntzen *et al.* (1) reported that stomatal closure was involved in an effect of toxin on photosynthesis and transpiration of Tms corn. They indicated that the specific action of toxin was similar to that of ABA. Dark fixation of CO_2 should be less affected than photosynthesis if stomatal closure is a major factor. Table II shows the reverse to be true. In this experiment, dilution of a crude extract of toxin caused 50% inhibition of dark fixation of CO_2 at 1/11,000, while 50% inhibition of photosynthesis required a dilution of 1/1200. The rate of photosynthesis was 5.5 mg CO_2 dm^{-2} hr^{-1} or 1 mg hr^{-1} on the basis of 1 mg of Chl. Further, the action of toxin on Tms corn of both processes parallels the effect of HPMS, another compound known to induce stomatal closure, rather than ABA (Table II). Arntzen *et al.* (1) reported effects in less than 60 min with excised leaves allowed to take up toxin in the transpiration stream. Shorter duration of treatment reduces the effectiveness of race T toxin, but the differential inhibition of dark fixation of CO_2 by HPMS on both Tms and N corn still is observed with only 1 hr of treatment of leaf discs (Table III).

An obvious site for action of toxin in inhibiting both dark and light fixation in corn is PEP carboxylase, but dilutions of 1/30 or 1/300 did not directly inhibit PEP carboxylase prepared from Tms leaves when added directly to the assay mixture. Preincubation of enzyme extract with toxin in ratios of 1:2 for as long as 100 min had no measurable effect. Ting and Osmond (27) have described kinetic differences among

Table I. *Effect of Length of Light Pretreatment in Presence of Toxin on Percentage Inhibition of Dark Fixation of CO_2 by Discs of W64A-Tms Corn Leaves*

Duration of Light Pretreatment	Inhibition	
	1/500 Dilution	1/5000 Dilution
hr	%	
1	56	14
3	78	34
5	88	51
7	91	61
9	89	69

alloenzymes of PEP carboxylase. It is possible that the differential effects of toxin on photosynthetic and dark fixation of CO_2 (Tables II and III) might be due to differential sensitivities between a photosynthetic C_4 carboxylase (27) and a carboxylase functioning specifically in dark fixation by leaves. Since carboxylase prepared from Tms roots was not affected by toxin, this possibility does not seem likely. It has been reported that HPMS can inhibit PEP carboxylase, but with the procedures used the inhibition was not appreciable, nor did ABA have a significant effect. In a separate experiment, excised leaves were placed in 10 mM KH_2PO_4 with and without 1/100 toxin for 5 hr, but the activity of PEP carboxylase was not affected by toxin.

The usefulness of inhibition of dark fixation of CO_2 as a bioassay is indicated in Table IV and in Figure 3. Table IV gives calculated dilution values for 100 and 50% inhibition of several different preparations of toxin each assayed from 1 week to several months after storage at −20 C. The slopes are included because they can be an important measure of relative sensitivity independent of the value for 50% dilution. In assays conducted for other purposes and not shown in Table III, toxin prep A showed additional values of 11,500, 11,500,

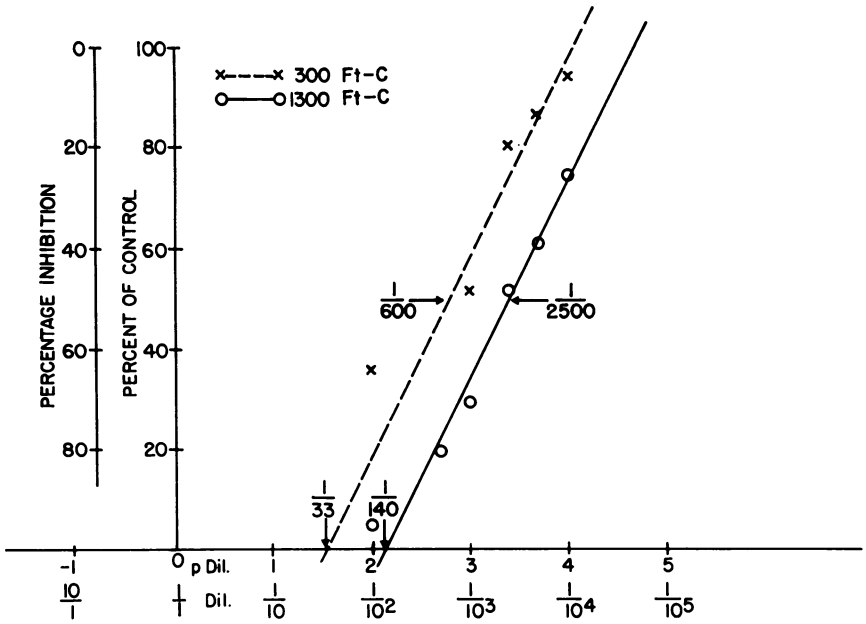

FIG. 2. Effect of incubation under light on the inhibition of dark CO_2 fixation in susceptible leaves of corn. Leaf discs were exposed to various dilutions of toxin for 30 min at a light intensity of 1300 ft-c. After rinsing with distilled H_2O, the discs were incubated without toxin for 6.5 hr at light intensities of either 300 (×) or 1300 (○) ft-c. Dark $^{14}CO_2$ fixation was carried out at 0.1% CO_2 for 1 hr.

Table II. *Effect of Toxin, ABA, and HPMS on $^{14}CO_2$ Fixation by Tms Corn Leaf Discs in Darkness or With 2500 ft-c Fluorescent Light*

Discs were preincubated for 6 hr in light followed by fixation for 50 min at approximately 0.1% CO_2 in a total volume of 1.6 liters.

Toxin Dilution	Inhibition and Standard Deviation	
	Dark	2500 ft-c
	%	
1/100		96.6 ± 0.1
1/250		90.2 ± 4.9
1/500	95.8 ± 0.6	84.8 ± 3.2
1/1000	92.6 ± 2.2	58.9 ± 8.7
1/2500	81.5 ± 4.1	21.1 ± 10.7
1/5000	61.6 ± 4.6	
1/10,000	49.5 ± 4.5	
ABA		
1.0 mM	62.7 ± 9.1	90.9 ± 3.5
0.1 mM	17.0 ± 7.5	74.7 ± 5.5
HPMS		
1.0 mM	58.9 ± 3.8	32.1 ± 10.1
0.1 mM	8.4 ± 9.5	−13.2 ± 4.6

Table III. *Comparison $^{14}CO_2$ Fixation after 1 Hr Treatment of Discs from Tms and N Leaves with Toxin, ABA, and HPMS*

The percentage inhibition was the average of two values.

Treatment	Inhibition of CO_2 Fixation in			
	Dark		1300 ft-c light	
	Tms	N	Tms	N
	%			
1/100 toxin	62	7	32	2
ABA				
1.0 mM	50	38	83	78
0.1 mM	5	9	64	68
0.01 mM	9	8	29	44
HPMS				
1.0 mM	52	60	27	37
0.1 mM	13	9	−5	

11,200, 9,700, and 9,800 for 50% inhibition. Figure 3 indicates that innate differences in the sensitivity even of different lines or varieties with Tms cytoplasm may be detectable. The slopes of the regression lines of B37-Tms and Mo17-Tms are different from W64A-Tms as is the dilution causing 50% inhibition of fixation. In this test, dilutions of 1/100 to 1/250, rarely used in assays with W64A-Tms, were included because of the relative insensitivity of B37-Tms and Mo17-Tms that had been observed during other comparisons with W64A corn.

Figure 4 presents one of the better results of a toxin bioassay based on root growth inhibition. A comparison with Figure 1 indicates that root growth is much less sensitive to toxin than is dark CO_2 fixation. The same toxin was used and the assays were conducted only 1 or 2 days apart. Table V shows the best four results of root assays conducted over a period of several weeks. Several other attempts were discarded for various reasons, including microbial contamination. Even the best results show more variation than does a routine dark fixation assay (compare with Table IV).

In the course of these experiments, we have tried bioassays based on fresh weight or Chl loss over several days exposure to toxin, as well as the loss of ^{14}C metabolites from preloaded discs. Although the procedures can be made quantitative, the former are time-consuming. The latter is less reliable than dark CO_2 fixation and less sensitive. With toxin preparation A, only dilutions of 1/500 or less induced leakage in the first few hr and 20 hr treatment caused significant leakage at dilutions of 1/2500 and 1/5000.

An unexpected aspect of the inhibition of dark CO_2 fixation by race T toxin was the effect of light (Fig. 2). Potentiation of

toxin action by light has not been reported, but influences of light on visual symptoms have been observed in our laboratory. For Figure 5, leaves were wrapped in Saran wrap to limit transpirational losses and alternate sections were banded with aluminum foil before immersion of cut ends into toxin for 72 hr. The darkened (D) areas of Tms corn retained Chl, whereas areas exposed to light (L) were completely bleached. It was shown that toxin is present in the sections which retained Chl by the fact that these sections bleach rapidly when excised and exposed to light in the absence of additional toxin. As illustrated in Figure 5, darkened sections of toxin-treated Tms leaves often appeared to retain more Chl than darkened portions of toxin-treated N or untreated T and N leaves.

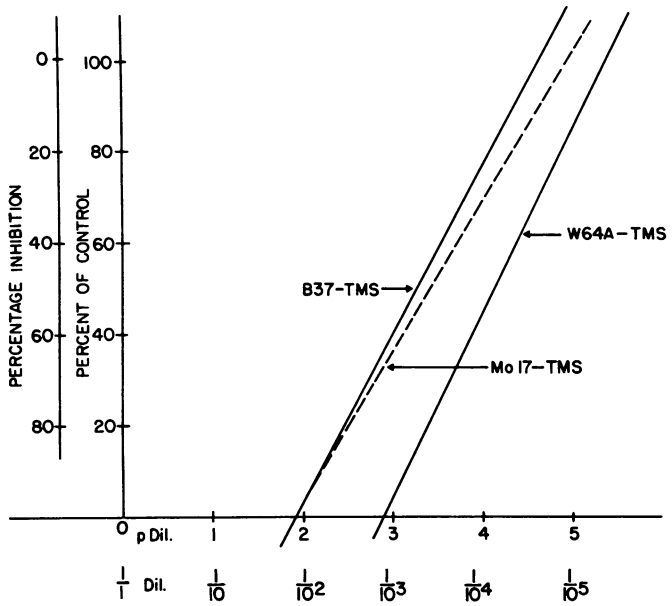

FIG. 3. Comparison of race T toxin inhibition of dark CO_2 fixation by three corn cultivars with Tms (susceptible) cytoplasm.

Table IV. *Dark CO_2 Fixation Assay of Different Preparations of Race T Toxin*

Prep	Calculated Dilution		Correlation Coefficient	Slope of Line
	100% inhibition	50% inhibition		
A[1]	1/300	1/11,000	0.97	31.6
A	1/310	1/9400	0.99	33.8
A	1/280	1/11,000	0.98	31.5
Avg	1/300	1/10,500		
B[2]	1/200	1/8500	0.94	30.8
B	1/240	1/8200	0.97	32.5
B	1/140	1/6000	0.95	30.4
Avg	1/200	1/6900		
C[3]	1/340	1/13,000	0.96	31.5
C	1/400	1/18,000	0.94	30.5
Avg	1/370	1/15,500		

[1] Preparation A assayed 8/13/73, 8/23/73, 5/27/74.
[2] Preparation B assayed 4/8/74, 5/27/74, 6/17/74.
[3] Preparation C assayed 6/6/74, 6/17/74.

Table V. *Calculated Dilution Values at 50% and 100% Inhibition of Corn Root Growth in Different Assays of Preparation A of Race T Toxin*

Assay	Calculated Dilution		Correlation Coefficient	Slope of Line
	100% inhibition	50% inhibition		
1	1:59	1:3070	0.86	29.1
2	1:51	1:1240	0.89	36.0
3	1:42	1:3030	0.91	27.0
4	1:41	1:1640	0.87	31.3

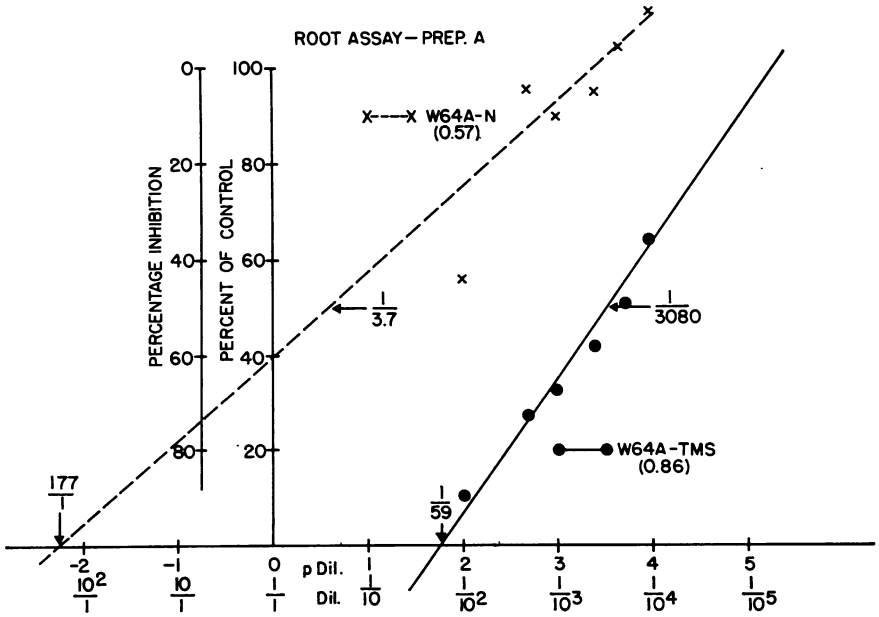

FIG. 4. Inhibition of corn root growth by race T toxin, preparation A. Resistant (×); susceptible (●). Same conventions as in Fig. 1.

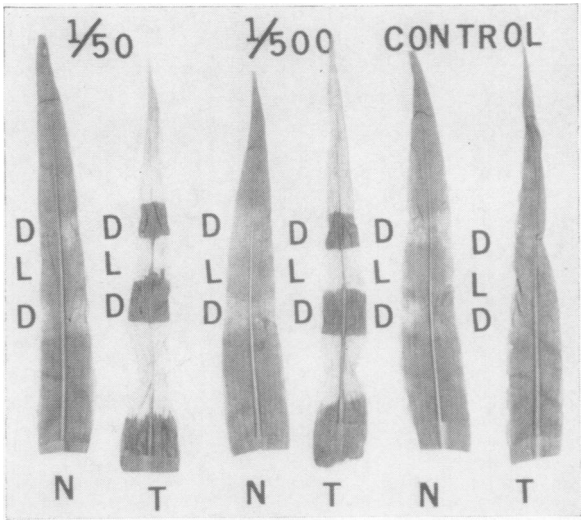

FIG. 5. Effect of light (2500 ft-c) on symptom development of toxin treated leaves of W64A Tms corn. Leaves banded with aluminum foil (D) or unbanded (L) with cut ends immersed in toxin for 72 hr.

DISCUSSION

For the processes we have compared directly with equivalent toxin preparations, dark fixation of CO_2 consistently has been the most sensitive, perhaps because it is affected through an action of toxin that is different from the action responsible for other measurable responses. Arntzen *et al.* (1) pointed out that, even though toxin does affect mitochondria (13), it was difficult to ascribe to mitochondria a role in the closure of stomates of Tms corn treated with toxin. Similarly, it seems unlikely that inhibition of dark CO_2 fixation is due to stomatal closure because of the differential effects of toxin on dark and light CO_2 fixation. As a mechanism of action, stomatal closure seems inconsistent with the rapid wilting of Tms leaves caused by race T toxin. It is possible that stomates initially close, but subsequently open, thus leading to wilt.

Attempts under our conditions (Tables II and III) to compare toxin with compounds known to close stomates were inconclusive and, in fact, the data with ABA and HPMS are difficult to explain. It is known that HPMS affects processes other than stomatal closure, including inhibition of PEP carboxylase and glycolate oxidation (15). It is surprising, therefore, to find a greater inhibition by HPMS of dark fixation rather than photosynthesis in a plant having a C_4 pathway of carbon reduction. However, the leaf carboxylase of W64A corn is only slightly inhibited by HPMS, and HPMS is reported to inhibit photosynthesis of C_3 plant chloroplasts essentially devoid of PEP carboxylase (14).

The inhibition of dark CO_2 fixation of susceptible lines of corn is approximately 3 times more sensitive to race T toxin than is inhibition of root growth (Figs. 1 and 4). In studies to be presented elsewhere, we have found that as little as 0.02 μg/ml of a purified toxin preparation will cause 50% inhibition of dark fixation. In addition to its importance in the choice of a useful bioassay system, the sensitivity of dark CO_2 fixation relative to other processes affected by the toxin has a bearing on possible modes of action. If the toxin affects a primary site which then causes secondary disturbance of dependent events, the secondary disturbance should be observed at dosages no greater than those required for the primary act. In addition, the slopes of the dilution curves should be similar for the primary and secondary effects.

Inhibition of mitochondrial oxidation is observed at reported dilutions of 1/20 to 1/100 but there did not appear to be a decrease in inhibition with dilution over that range (13). It is not clear whether the dilutions were based on the toxin content of the original culture filtrates as we have done. In subsequent papers (1, 2) the crude filtrate first was concentrated 10-fold. In other studies with mitochondria (9, 16), preincubation with dilutions of 1/100 apparently were required for rapid inhibition. Similarly, it appears that high levels of toxin are required for the specific inhibition of an ATPase from Tms corn (28). Although the protocol in that paper is not clear, a companion article (7), indicates that the same toxin routinely was used at concentrations equivalent to 1/10 dilutions of original filtrate.

The high level of toxin required in those studies is not expected for essentially enzymatic processes, with no significant diffusion barriers. The effects observed are very rapid, however, in contrast to the longer periods required for substantial inhibition of dark CO_2 fixation or for ion leakage. In the latter instance, 1/10 dilutions of race T toxin caused significant increases in leakage only after 1 (7) and 3 (6) hr exposure to toxin.

The minimum time required to complete a dark CO_2 fixation assay was 16 min. At dilutions of 1/20 and 1/100 and with only a 5-min light pretreatment, the percentage inhibition was 50 and 30%, respectively. Although inhibition of dark CO_2 fixation undoubtedly is a secondary effect of toxin, the dilutions required for rapid action are of the same order of magnitude as for reactions that are suggested to be more directly involved in toxin action.

We routinely have used lesion formation on excised leaves (Fig. 5) as a quick measure of relative toxicity since dilutions of 1/1000 will cause severe lesions within 16 hr while dilutions of 1/50,000 cause similar effects by 72 hr. Karr *et al.* (8) also have used a leaf lesion assay for race T toxin which was based on a leaf puncture method described by Steiner and Strobel (24) for a host-specific toxin affecting sugarcane. Karr *et al.* (8) reported that a 5-μl drop containing 10^{-8} moles of race T toxin was used in each assay. If the purity and postulated mol wt of 388 daltons (8) is accepted, this corresponds to a toxin concentration of 776 μg/ml. Since apparently 90% of the biological activity was recovered as 600 μg of material extracted from 1 liter of culture filtrate, 776 μg/ml represents a 1100-fold concentration of toxin, rather than to a dilution. Each 5-μl drop would be equivalent to 5.8 ml of original culture filtrate.

Because our toxin preparations might be different, the procedures of Karr *et al.* (8) were followed and were found to be insensitive, requiring 10- to 20-fold concentration of toxin to produce a lesion of approximately 1 cm. For a similar leaf puncture assay, Steiner and Byther (23) apparently used 5-fold concentrations of sugarcane host-specific toxin from *H. sacchari*. Steiner and Strobel in two papers (24, 26) presented a standard bioassay curve for the *H. sacchari* toxin which indicated that a purported concentration of 10^{-8} moles caused a lesion 9 cm long. Since the leaf puncture assays for both sugarcane and race T toxins are comparable, the lesion length would indicate that the sugarcane-*H. sacchari* system has greater sensitivity than the corn-*H. maydis* system or that the toxin diffuses more rapidly. In a later paper (25), however, the response to 10^{-8} moles of *H. sacchari* toxin, calculated as the average of five replicates, ranged from 2 to 12 cm in seven determinations, considerable variation for a quantitative bioassay. The range among the replications was not indicated.

The choice of a suitable bioassay for host-specific toxins obviously will depend on the particular system under investiga-

tion. Victorin, for example, does not appear to affect mitochondrial oxidation (3), while a host-specific toxin of *H. carbonum* stimulates (10), rather than inhibits, dark CO_2 fixation of corn. Inhibition of dark CO_2 fixation has been useful in quantitatively monitoring purification procedures for race T toxin and may have considerable utility in distinguishing differences among lines of corn with Tms cytoplasm (Fig. 3). In this respect, there has been no reported success so far with other bioassays which are available.

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