Comparative Effects of Host-Specific Toxins and Helminthosporium Infections on Respiration and Carboxylation by Host Tissue

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ABSTRACT

Oxygen uptake and CO_2 fixation in the dark were used as parameters to compare the response of corn to *Helminthosporium carbonum* (HC) and to its host-specific toxin. Further comparisons were made with the effects of *H. victoriae* (HV) toxin on susceptible oats. *Helminthosporium carbonum* infection and HC-toxin caused small increases in respiration of corn leaves. *H. carbonum* and its toxin, as well as HV-toxin, increased the capacity of susceptible tissues to fix CO_2 in the dark. Cell-free preparations from HC-toxin-treated corn leaves fixed more CO_2 with ribose-5-P as the substrate than did the control from nontreated tissues. No increase in CO_2 fixation was evident when HC-toxin was added to extracts from nontreated corn tissues. The data, plus previously published genetic studies, show that HC-toxin is as significant in pathogenesis and host-specificity of *H. carbonum* as is HV-toxin to *H. victoriae*. Phytopathology 60:1391-1394.

Additional key words: Helminthosporium carbonum, H. victoriae, Avena sativa, Zea mays.

Pathogenicity by some plant-infecting fungi clearly depends on production of substances selectively toxic to host plants (15). For example, the determinant of pathogenicity of *Helminthosporium victoriae* Meehan & Murphy, HV-toxin, is extremely toxic to susceptible oats (*Avena sativa* L.) (14), is somewhat toxic to tolerant oats (17), and has little effect on resistant oats and nonhost plants (15) unless used in massive concn. Another example is *H. carbonum* Ullstrup race 1, which infects certain kinds of dent corn (*Zea mays* L.). Its determinant, HC-toxin, is highly toxic to susceptible corn, less toxic to corn intermediate in the susceptible-to-resistant scale, and still less toxic to resistant corn (5).

The primary purpose of this work was to evaluate the role of HC-toxin in disease development, by comparing the effects of *H. carbonum*-infection with the effects of HC-toxin on corn tissues. A second aim was to compare some physiological effects of HC- and HV-toxins on sensitive plants. Susceptible but not resistant oat tissue is known to respond quickly to HV-toxin with increased respiration (14). The ability of HC-toxin to induce a similar response in corn was determined. Experiments with ^{14}C-carbon dioxide were based on the work of Malca et al. (6), who found that *H. carbonum* infection first increased and then decreased the potential ability of tissues to fix CO_2 in the dark.

The initial effect of HV-toxin is believed to be on the plasma membrane (12). An analysis of the effects of HC-toxin on cell permeability is underway, and will be reported later.

MATERIALS AND METHODS.—Corn hybrids Pr × K61 and Prl × K61 were used; Pr × K61 is susceptible and Prl × K61 is resistant to *H. carbonum* race 1 and to HC-toxin. The hybrid seeds were supplied by A. J. Ullstrup, Purdue Univ. Corn seeds were surface-sterilized with 0.5% sodium hypochlorite (w/v) for 2 hr, then washed thoroughly and germinated on moist filter paper. When small seedlings or coleoptiles were needed, plants were grown in White's solution in petri dishes. When more mature tissue was needed, treated seeds were either grown in the laboratory in vermiculite watered with nutrient solutions, or in the greenhouse in sand:soil:peat mixture (1:1:1) watered as needed with a soluble commercial fertilizer. Shoots were taken as cuttings from 10- to 14-day-old plants with three or four expanded leaves. Unless otherwise stated, the first leaf above the primary leaf was used. Oat leaves from plants susceptible (cv. Park) and resistant (cv. Clinton) to *H. victoriae* infection and to HV-toxin were used as required.

Spores from 10-day-old cultures grown on V-8 juice agar were used for inoculum. Preparation of inoculum and methods of inoculation were described previously (13). HC-toxin was isolated from culture filtrates of a high-yielding strain of *H. carbonum*, using gel filtration methods as described previously (4). The more active preparations completely inhibited growth of susceptible seedlings at 1.1 μg/ml, an activity which approaches that of crystalline toxin (10). The standard seedling bioassay for HC-toxin (13) was used. HV-toxin isolation and bioassay also followed previously described methods (9, 12). Both toxins are unstable, and each preparation differed somewhat in relative activity. Therefore, the minimum concn which gave complete inhibition of susceptible seedling root growth is stated for each experiment.

Standard manometric techniques were used to determine gas exchange (16). Cuttings from corn plants were allowed to take up toxin solutions for specified times. Following toxin treatment, 5-cm sections were cut from the centers of leaves and the midrib was discarded. Leaf tissue (0.15 to 0.2 g fresh wt) was placed on moist filter paper in each Warburg flask. In some experiments with roots, tissues were pretreated

with toxin and placed on moist filter paper in Warburg flasks. In other experiments, roots were placed in flasks containing 2 ml White's solution with or without toxin. Six carefully selected roots were used in each flask. *Helminthosporium carbonum*-infected leaves were required in some experiments. In these cases, the numbers of spores deposited on leaves was determined by microscopic observation; the inoculated and control plants were held in a fog chamber for 24 hr. Uniformly inoculated leaves were selected from an equivalent position on each plant, and sections were cut from the middle of each leaf. Experiments with intact tissues were at 25 C.

Toxin-treated and nontreated leaves of corn or oats were allowed to fix $^{14}CO_2$ in the dark for 3 to 4 hr. Similar experiments were done with fungus-infected and noninoculated leaves. $^{14}CO_2$ was generated in a 1,000-ml sealed glass chamber by adding excess lactic acid to ^{14}C-sodium bicarbonate (25 μc). After dark fixation, leaves were removed from the chamber and washed first with 0.5 N HCl and then with distilled water to remove excess $^{14}CO_2$ adsorbed on the leaf surface. Washed leaves were blotted dry, cut into small pieces, and dried for 24 hr at 80 C. Dried samples were degraded to CO_2 by wet combustion, using 5 ml liquid and 1.0 g solid van Slyke reagent/14 mg dry samples (1). The $^{14}CO_2$ released was trapped in an absorption flask containing 15 ml 0.1 N NaOH. Aliquots (0.1 or 0.2 ml) of the NaOH solution were placed on sand-blasted glass planchets, dried, and counted in a windowless gas flow detector.

Clarified plant homogenates were used in experiments on dark fixation of CO_2. Leaf tissue (3 g fresh wt) was cut into small pieces with scissors and ground with sand in 15 ml 0.5 M Tris [tris(hydroxymethyl) amino methane] buffer (pH 8.0) in a mortar at 2 C. The resulting slurry was pressed through several layers of cheesecloth and centrifuged at low speed to remove debris. The supernatant was centrifuged again at 35,000 *g* for 20 min and finally at 105,000 *g* for 2 hr. The final supernatant of clear amber-colored fluid was used as an enzyme source.

Enzymatic assay methods were essentially those of Malca et al. (6). The enzyme preparation (0.3 ml) was added to the reaction mixture (0.7 ml) and incubated at the appropriate temp and times before the reaction was stopped by addition of 1.0 N HCl (0.1 ml). Aliquots of the reaction mixtures were placed on sand-blasted glass planchets and dried for radiation counts. Reaction mixtures contained, in addition to enzyme preparation, the following, in μmoles: $MgCl_2$, 15; $NaH^{14}CO_3$, 5 (containing 2.5 μc); Tris buffer at pH 8.0, 150; adenosine-5-triphosphate (ATP, disodium salt), 2; and substrate, 1 or 2. The following substrates, from Sigma Chemical Co., were used for the carboxylation reactions: D-ribose-5-phosphate (disodium salt); D-ribulose-1,5-diphosphate (tetrasodium salt); phosphoenolpyruvate (trisodium salt); and D (-)2-phosphoglycerate (tricyclohexylammonium salt). Further details in the assays are described by Malca et al. (6). All assays were duplicated or triplicated and all experiments repeated one or more times.

RESULTS.—*Effect of H. carbonum infection and HC-toxin on tissue respiration.*—Resistant and susceptible corn plants with 4 or 5 expanded leaves were inoculated with *H. carbonum* spore suspensions. The surface of inoculated leaves had 8-10 spores/mm^2, as determined by microscopic observation. Leaf samples were cut at daily intervals for determination of gas exchange; noninoculated control leaves in a typical experiment took up 550 μliters O_2/g (fresh wt)/hr. There were no differences in oxygen uptake by inoculated and control leaves 24 hr after inoculation. At 48 and 72 hr after inoculation, respiration was approximately 30% higher in inoculated than in control leaves; thereafter, respiration in diseased tissue gradually decreased. Increased oxygen uptake in this case cannot be attributed entirely to host tissue, because fungus was present. Respiration rate was not affected in inoculated resistant leaves.

Cuttings of corn plants were allowed to take up HC-toxin (5 μg/ml) for varying periods of time. The toxin preparation completely inhibited root growth of susceptible seedlings at 5.0 μg/ml. Sections of leaves were cut from treated and control plants, and oxygen uptake by the leaf sections was measured. Leaves from control plants took up 35-43 μliters oxygen/hr per 10 mg dry wt in 3 experiments. Exposures to toxin of 2 and 4 hr caused no increase in respiration. Eight-hr exposure caused a 30% increase in oxygen uptake; higher uptake by treated than by control tissue was still evident 30 hr after exposure to toxin. There was no visible damage to treated leaves at the time oxygen uptake was measured, but visible necrosis occurred by 48 hr after treatment. Another toxin preparation which gave complete inhibition of root growth at 4.5 μg/ml had no effect on oxygen uptake by susceptible leaves at 0.25 and 1.0 μg/ml. Leaves treated for 5 hr with this preparation at 4.5 μg/ml had 20% more oxygen uptake than did control leaves, which used 41 and 46 μliters oxygen/hr per 10 mg dry wt in 2 experiments.

A test was made for quick respiratory responses of roots to HC-toxin, for comparison with the effects of HV-toxin on oats (14). Oxygen uptake by roots from susceptible seedlings exposed to HC-toxin was determined in Warburg flasks containing 2.0 ml White's solution with 50 μg toxin/ml. Respiration of roots was not affected over a 2-hr period.

Oat leaves from plants inoculated with *H. victoriae* take up more oxygen than control leaves within 3 days (11). HV-toxin stimulates gas exchange in susceptible tissue within min (14). Thus, respiratory increase caused by HC-toxin is less striking and develops much more slowly than that caused by HV-toxin.

Effects on dark fixation of CO_2.—We repeated the $^{14}CO_2$ fixation experiments of Malca & Zscheile (7), using a wide range of inoculum concn and exposure times. $^{14}CO_2$ fixation was decreased by infection in all cases; the values ranged from 65 to 83% of control values, which were comparable to the controls listed in Table 1. Some reduction was evident as early as 24 hr after inoculation. Comparable reduction in CO_2 fixation was caused by inoculation of corn with the oat pathogen *H. victoriae,* which penetrates the corn leaf

TABLE 1. Effect of *Helminthosporium carbonum*-toxin on $^{14}CO_2$ dark fixation by corn leaf tissue

Tissue type	Toxin concn	CO_2 fixed	Increase
	μg/ml[a]	CPM[b]	%
Susceptible	0	490	
	5.0	706	44
Susceptible	0	464	
	50.0	1,379	197
Resistant	0	361	
	50.0	485	34

[a] Cuttings were exposed to toxin solutions for 4 hr in the light before exposure for 4 hr in the dark to $^{14}CO_2$. Toxin preparation completely inhibited growth of susceptible seedling roots at 2.0 μg/ml.

[b] Average counts per min per planchet, above background, for duplicate or triplicate samples. The data are from three separate experiments.

surface but fails to develop further. In addition, corn cuttings allowed to take up $CuSO_4$ (5×10^{-4} M) for 4 hr fixed 30-40% less CO_2 in darkness than did nontreated controls. Apparently, various kinds of injuries can decrease CO_2 fixation. In contrast, Malca et al. (6) have shown that infection increases the potential for dark fixation of CO_2, even though actual fixation is reduced. Stimulatory and inhibitory components must be involved in infection.

Cuttings from resistant and susceptible corn plants were used to determine the effect of HC-toxin on dark fixation of CO_2. After cuttings took in toxin solutions (5 or 50 μg/ml) for 4 hr, leaf sections were cut and exposed to $^{14}CO_2$ for another 4 hr. Tissues were then combusted to CO_2 and the radioactivity was measured. Under the conditions of this experiment, toxin caused a striking increase in CO_2 fixation by susceptible leaves, and a slight increase in resistant leaves. The results shown in Table 1 are representative of a number of experiments. The difference in stimulation in resistant and susceptible leaves appears to reflect the difference in response to toxin as measured by other parameters. Toxin stimulation of CO_2 fixation became less evident with longer exposure times, until treated tissues fixed less CO_2 than did controls. When toxin-treated cuttings were placed in darkness for 2 hr before exposure to $^{14}CO_2$, the fixation was only slightly higher (+16%) than that of controls. In this same experiment, toxin-treated tissue without the dark period fixed 90% more CO_2 than control without toxin. Similar differences were reported by Daly & Livne (2) for CO_2 fixation by rust-infected leaves.

TABLE 2. Effect of *Helminthosporium victoriae* toxin[a] on $^{14}CO_2$ dark fixation by susceptible oat leaves

Treatment	Exposure time[b]	$^{14}CO_2$ fixed	Change
	hr	CPM[c]	%
Control	0	147	
Toxin (0.8 μg/ml)	0.5	157	+ 7
Control	0	117	
Toxin (0.16 μg/ml)	4	158	+35
Control	0	123	
Toxin (0.32 μg/ml)	4	222	+81

[a] Toxin preparation completely inhibited root growth of susceptible seedlings at 0.016 μg/ml.

[b] Followed by 4-hr incubation with $^{14}CO_2$.

[c] Counts per min (above background) per planchet. Total CPM fixed by the whole preparation was 75 times these values. Each experiment was duplicated.

The effect of HV-toxin on dark fixation of CO_2 was determined with *H. victoriae*-susceptible and resistant oat cuttings. The toxin preparation gave complete inhibition of susceptible seedling root growth at 0.016 μg/ml. CO_2 fixation by the susceptible leaf was not affected after 30-min exposure to toxin (0.8 μg/ml) followed by 4-hr incubation with $^{14}CO_2$. Susceptible cuttings exposed to toxin (0.32 μg/ml) for 4 hr, followed by 4-hr incubation with $^{14}CO_2$, had 81% more CO_2 fixation than did the respective nontreated control tissues (Table 2). HV-toxin had no effect on CO_2 fixation by resistant oat leaves.

The effects of HC-toxin on carboxylation mechanisms were examined with cell-free preparations. Susceptible corn cuttings were allowed to take up toxin (5 μg/ml) for 4 hr, and held for another 4 hr in the dark at 22 C. Leaves were then ground and the resulting homogenate was filtered and centrifuged. The supernatant was used as an enzyme source in a reaction medium including $H^{14}CO_3$. Several carboxylation systems were tested, using appropriate substrates and cofactors. Toxin had little or no effect on the phosphoenolpyruvate and 3-phosphoglycerate systems. Erratic results were obtained with the ribulose-1,5-diphosphate system in several experiments. With ribose-5-phosphate as the substrate, extracts from toxin-treated plants in 4 experiments had 61, 47, 99, and 89% more CO_2 fixation than did extracts from control plants (Table 3). Similar results were reported by Malca et al. (6) using extracts of plants obtained shortly after inoculation with relatively low concn of *H. carbonum* spores.

In further experiments, cuttings were not treated with toxin; instead, toxin was added to enzymes extracted from nontreated tissues. Before addition of

TABLE 3. Carboxylation activity of cell-free extracts from *Helminthosporium carbonum*-toxin (5 μg/ml) treated tissue

Reaction system[a]	$^{14}CO_2$ fixed[b]	
	Controls	Toxin-treated
	CPM	CPM
Ribose-5-P, ATP, CO_2, Mg++	448	848[c]
Mg++, CO_2, ATP	30	30
Ribose-5-P, Mg++, CO_2	66	44
Ribose-5-P, ATP, Mg++, boiled enzyme	−2	−2

[a] Total volume of reaction mixture was 1.0 ml, including 0.3 ml supernatant enzymes. Other components (in μmoles) were: $MgCl_2$, 15; $NaHCO_3$, 5 (with 25 μc $H^{14}CO_3^-$); Tris [tris (hydroxymethyl) amino methane] buffer (pH 8.0), 150; adenosine triphosphate, 2; and ribose-5-phosphate, 2.

[b] Counts per min, above background, for comparable samples. Toxin preparation completely inhibited root growth at 1.1 μg/ml.

[c] Increase = 89%. Toxin caused 61, 47, and 99% increased CO_2 fixation in three other experiments.

labeled bicarbonate, HC-toxin at 0.5, 2.5, or 5.0 μg/ml was added and the reaction mixture was incubated for 30 min. Toxin did not affect $^{14}CO_2$ uptake by the ribose-5-phosphate system. The increase in dark fixation of CO_2 therefore appears to be a secondary effect of toxic action.

DISCUSSION.—Increased respiration is a usual response of plants to infection by bacteria and fungi. This typical response can be elicited by both *H. victoriae* and *H. carbonum* infections. The toxins produced by both fungi also cause respiratory increases, although they differ in the speed and magnitude of stimulation. *Helminthosporium victoriae* infection and HV-toxin caused a large increase in gas exchange by susceptible oat tissues (11, 14, 15); *H. carbonum* infection and HC-toxin caused a relatively weak response in corn. This response was so slow to develop that we originally reported no effect of toxin on respiration (3). Previous data showed that respiratory stimulation by HV-toxin probably is a secondary event in its action (14, 15).

Malca et al. (6) showed that extracts taken from *H. carbonum*-infected tissue before symptoms were evident had greater capacity to fix CO_2 in the dark than did control extracts. As symptoms appeared, the capacity to fix CO_2 fell below the control level. Circumstantial evidence indicated that inhibitors accumulated in the tissues as injury developed. When inhibitors were removed, the ability to fix CO_2 was restored to a level higher than that of controls (6). Our data indicate that HC-toxin is the stimulus for increased capacity of infected tissues to fix CO_2. Other factors must be responsible for accumulation of inhibitor, which apparently is induced by various types of injury. For example, inoculation of corn with an oat pathogen (*H. victoriae*), which penetrates the corn leaf surface but does not develop further, also caused a decrease in CO_2 fixation. When all these findings are considered, it seems evident that *H. carbonum* infection and HC-toxin have parallel effects on the capacity of tissues to fix CO_2 in the dark.

HC-toxin also caused increased CO_2 fixation in resistant corn leaves, but the increase was much less than in susceptible leaves at a given toxin concentration. HV-toxin stimulated dark fixation of CO_2 by susceptible oat leaves, but relatively high concn had no effect on resistant oats. Similar stimulations in CO_2 fixation in the dark following infection by rust fungi has had much study (2, 8).

An attempt was made to determine the reactions involved in toxin-induced CO_2 fixation in order to compare more precisely the changes induced by HC-toxin and infection. Several compounds known to be substrates in CO_2-fixing systems were added to enzyme extracts from control plants and to extract from plants previously treated with HC-toxin. Maximum toxin-induced stimulation of CO_2 fixation occurred with ribose-5-P as the substrate. Again, the effects of HC-toxin and infection were the same (6). The indications are that both toxin and infection increase the capacity for dark fixation of CO_2 by affecting the activity of phosphoribulokinase, or phosphoribosisomerase, or both. But this appears to be another secondary effect of toxin, since CO_2 fixation did not change when toxin was added to a cell-free extract rather than to tissues before extracts were made.

It is evident from these and previous findings (4, 5, 10, 13) that HC-toxin is as important to infection by *H. carbonum* as HV-toxin is to *H. victoriae*. There are nevertheless some quantitative and qualitative differences in their actions.

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