Comparative Specificity of the Toxins of Helminthosporium carbonum and Helminthosporium victoriae

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ABSTRACT

A toxin preparation from *Helminthosporium carbonum* (HC) caused 50% inhibition of seedling root growth of a *H. carbonum*-susceptible corn hybrid at 1.0 μg/ml, but caused only 13% inhibition of a near-isogenic resistant hybrid at 25.0 μg/ml. This preparation stimulated root elongation of susceptible corn at 0.125 and resistant corn at 3.125 μg/ml. Inbred corn lines that are low, intermediate, or high in resistance to *H. carbonum* had the same relative rank in sensitivity to HC-toxin. Of several plant species tested (barley, corn, cucumber, oats, radish, sorghum, tomato, and wheat), susceptible corn was the most sensitive to HC-toxin, while resistant corn was among the most tolerant. The sensitivity of several nonhost plants fell between these extremes. In contrast, a preparation of *H. victoriae* (HV) toxin completely inhibited root growth of susceptible oats at 0.009 μg/ml, but caused only partial inhibition of resistant oats and other plants (tomato, corn, wheat, sorghum, and barley) at 3,600 μg/ml. Thus, resistant oats and other nonhost plants will tolerate > 400,000 times higher concentrations of HV-toxin than will susceptible oats. HV-toxin inhibited growth of nonhost plants about the same as did methionine. Phytopathology 60:365-368.

Several plant pathogens are known to produce metabolites that are necessary for pathogenicity and that are more toxic to the natural host than to other plants (9, 13). Among these is the toxin of *Helminthosporium victoriae* Meehan & Murphy, which is specific to certain cultivars of oats (*Avena sativa* L.), and the toxin of *H. carbonum* Ullstrup race 1, which is selectively toxic to certain kinds of dent corn (*Zea mays* L.). These two substances are similar chemically (10), although they differ in specificity.

Both *H. carbonum* (HC) and *H. victoriae* (HV) toxins cause necrosis and inhibition of seedling growth of susceptible seedlings. Low concentrations of HC-toxin stimulate growth of corn seedlings (5), but similar effects of HV-toxin have not been reported. The main purpose of this study was to compare the inhibitory or stimulatory effects of HC- and HV-toxins on growth of host and nonhost plants. The data could help us to understand host-specificity of microorganisms, are potentially useful in comparing effects of toxins with effects of infections, and may serve as guides in evaluating other possible determinants of pathogenicity.

MATERIALS AND METHODS.—*H. carbonum*-resistant (Pr × K61) and susceptible (Pr × K61) corn hybrids were used for HC-toxin assays, and for other experiments with corn except as indicated otherwise. These hybrids and all corn inbred lines used were supplied by A. J. Ullstrup of Purdue University. The standard bioassay for HC-toxin (11), in which the endpoint was 50% inhibition of growth of susceptible seedling roots, was used. Oat cultivars susceptible (Park) and resistant (Clinton and Russel) to *H. victoriae* were used in experiments with HV-toxin, except as indicated otherwise. The standard bioassay for HV-toxin (9), in which the end point was complete inhibition of susceptible seedling root growth, was used.

HC-toxin was isolated from culture filtrates by a gel filtration procedure (6), and by the method of Pringle & Scheffer (10). Several different toxin preparations were used, including one supplied by R. B. Pringle of the Cell Biology Research Institute, Ottawa, Canada. Toxin preparations often differ in relative activity, because the toxin is unstable and preparations may differ in degree of purity. Therefore, toxicity per unit dry wt is given for each preparation. Toxin solutions from gel columns were assayed, while other aliquots of the same solutions were used for dry wt determinations after drying at 95 C for 48 hr. Highly purified preparations (10) were first weighed, then dissolved and assayed; this procedure can give a conservative estimate of toxin content because highly purified toxin is more unstable than toxin in crude preparations, and may be inactivated during the assay procedure.

HV-toxin eluted from an alumina column (9) was stable at pH 3.5 when stored at 4 C. Toxin was further purified for some experiments by the gel filtration method used with HC-toxin (6).

Toxins were tested against several plant species: barley (*Hordeum vulgare* L.); corn; cucumber (*Cucumis sativus* L.); oats; radish (*Raphanus sativus* L.); sorghum (*Sorghum vulgare* var. *subglabrescens* [Steud.] A.F. Hill); Sudan grass (*Sorghum vulgare* var. *sudanense* Hitchc.); tomato (*Lycopersicon esculentum* Mill.); wheat (*Triticum aestivum* L.); and ryegrass (*Lolium multiflorum* Lam.). Seeds of all species except corn were first germinated between sheets of moist filter paper at 22-30 C for 1 to 3 days, then placed in 60-mm petri dishes containing 5.0 ml White's inorganic solution (17) plus toxin at several concentrations. Corn seeds were placed embryo-down in 100-mm petri dishes containing 10 ml White's solution, and were incubated for 24 hr. Nutrient solutions were then replaced with White's solution containing toxin at ap-

propriate concentrations. Each dish contained five or six seeds, and each toxin concentration was used in 5-25 dishes. Control dishes contained White's solution without toxin. The longest root on each seedling was measured after 3 to 4 days in test solutions at approximately 22 C. Differences between means were detected by Duncan's multiple range test. Data are given for representative single experiments. All experiments were repeated two or more times with comparable results.

RESULTS.—*Effect of HC-toxin on growth of resistant and susceptible hybrid corn seedlings.*—Low concentrations of HC-toxin appeared to stimulate root growth in routine assays. Therefore, stimulatory and inhibitory effects of HC-toxin were evaluated on two near-isogenic corn hybrids resistant and susceptible to *H. carbonum*. The toxin preparation was used in concentrations from 0.0625 to 1.0 μg/ml for susceptible corn, and 3.125 to 25.0 μg/ml for resistant corn. Roots were measured after 78-hr incubation. With susceptible corn, toxin at 0.125 μg/ml caused a significant increase in root elongation, while 0.5 and 1.0 μg/ml caused significant inhibition (Table 1). This toxin preparation caused a significant increase in root elongation of *H. carbonum*-resistant corn at 3.125 and 6.25 μg/ml, and a decrease with 25.0 μg/ml (Table 2). Three other toxin preparations stimulated or inhibited resistant and susceptible corn, depending on concentration. In all cases, 25 to 50 times more toxin was required to stimulate resistant seedlings than was required for susceptible ones.

Comparative sensitivity of corn inbred lines and hybrids to HC-toxin.—Fourteen corn inbred lines and hybrids with known reactions to *H. carbonum* infection in the field (7, A. J. Ullstrup, *personal communication*) were tested for relative sensitivity to HC-toxin. The lines included three susceptible inbreds, one susceptible hybrid, one intermediate inbred, eight resistant inbreds, and one resistant hybrid. Seeds were treated with a series of toxin concentrations, and incubated at approximately 22 C for 4 days. Eight petri dishes were used for each toxin concentration. The toxin preparation used gave complete inhibition of susceptible hybrid (Pr × K61) seedling root growth at 1.1 μg/ml. Root growth of susceptible corn lines (K61, Pr, K44, and Pr × K61) was inhibited 64 to 75% by toxin at 0.6 μg/ml. The resistant lines (W37A, R61, Wf 9, P12170, Tr, GE 440, K61-1, K61-2, and Pr 1 × K61) were not affected significantly by toxin at 0.6 μg/ml. One inbred (P8), known to have intermediate resistance to *H. carbonum* race 1, was also intermediate in toxin sensitivity (37% inhibition of root growth by toxin at 0.6 μg/ml). Therefore, sensitivity to toxin was correlated with resistance and susceptibility to *H. carbonum* infection in the field.

Effect of HC-toxin on growth of nonhost plants.—Several gramineous plants were tested with a toxin preparation that completely inhibited root growth of the susceptible corn hybrid (Pr × K61) at 2.5 μg/ml. At 5.0 μg/ml, toxin had no effect on root growth of barley, wheat, grain sorghum, Sudan grass, ryegrass, oats, and corn hybrid Pr1 × K61.

A second series of experiments was designed to determine the effects of HC-toxin on root growth of several gramineous and dicotyledonous plants. The toxin preparation used in this experiment was from a gel column (6), and gave significant inhibition of susceptible and resistant corn root growth at 0.2 and 45.0 μg/ml, respectively. *H. carbonum*-susceptible corn was the most sensitive plant tested, while *H. carbonum*-resistant corn was among the least sensitive (Table 3). Radish, oats, and tomato were inhibited more than 40% by HC-toxin at 3.0 μg/ml; these plants are therefore more sensitive than resistant corn, but less sensitive than susceptible corn. At the lower concentrations, toxin caused increased root elongation in sorghum, barley, and tomato; the effect on sorghum was striking.

TABLE 1. Effect of *Helminthosporium carbonum* toxin on root growth by *H. carbonum*-susceptible hybrid (Pr × K61) corn seedlings

Toxin concentration (μg/ml)	No. of observations	Mean growth (mm)
0 (control)	91	111
0.0625	81	114
0.125	75	125[a]
0.25	91	110
0.5	96	80[a]
1.0	91	55[a]

[a] Significantly different ($P < .01$) from control.

TABLE 2. Effect of *Helminthosporium carbonum* toxin on root growth by *H. carbonum*-resistant hybrid (Pr 1 × K61) corn seedlings

Toxin concentration[a] (μg/ml)	No. of observations	Mean growth (mm)
0 (control)	97	120
3.125	97	129[b]
6.25	97	128[b]
12.5	97	119
25.0	97	104[b]

[a] Toxin preparation gave 50% inhibition of susceptible corn at 1.0 μg/ml.
[b] Significantly different ($P < .01$) from control.

TABLE 3. Effect of *Helminthosporium carbonum* toxin on root growth of host and nonhost seedling plants

Plant	Growth of controls	% Inhibition (−) or stimulation (+) at toxin concentrations (μg/ml)		
	mm	*0.2* μg %	*3.0* μg %	*45.0* μg %
Barley	37	− 2	+ 15[a]	− 44[b]
Corn, Pr 1 × K61	116	− 9	+ 6	− 66[b]
Corn, Pr × K61	114	−37[b]	−100[c]	−100[c]
Cucumber	58	+ 4	− 9	− 66[b]
Oat, cv. Park	54	− 4	− 13[b]	−100[c]
Oat, cv. Russel	74	+ 8	− 47[b]	− 80[b]
Radish	64	−15[a]	− 48[b]	− 81[b]
Sorghum	77	+34[b]	+ 40[b]	− 74[b]
Tomato	46	+15[b]	− 43[b]	−100[c]
Wheat	85	− 5	+ 1	− 53[b]

[a] Significantly different ($P = .05$) from control which had no toxin. All values are averages for 20 observations taken 80 hr after exposure to toxin.
[b] Significantly different ($P < .01$) from control.
[c] Roots < 10 mm and discolored.

The toxin preparation that gave some inhibition of susceptible corn at 0.2 μg/ml was inactivated by autoclaving (18), and retested against several *H. carbonum* host and nonhost plants. Inactivated toxin did not inhibit root growth of radish, wheat, tomato, and resistant corn seedlings at 40.0 μg/ml. It inhibited radish (43%) and wheat (24%), but not tomato and resistant corn at 400 μg/ml. Before inactivation, the toxin preparation drastically inhibited all species tested at 45.0 μg/ml (Table 3). Thus, the toxin molecule appears to have some nonspecific toxicity, and nonhost plants vary somewhat in sensitivity to it, as they do to other substances (see below).

Effect of HV-toxin on growth of H. victoriae-susceptible and resistant plants.—The HV-toxin preparation used to test the tolerance of oats (cultivars Park and Clinton), tomato, barley, wheat, grain sorghum, and corn completely inhibited root growth of Park oat seedlings at 0.009 μg/ml. All test species were partially or completely inhibited by the HV-toxin at 3600 μg/ml, but only Park oats were inhibited at lower concentrations (Table 4). Clinton oats were as tolerant of HV-toxin as any of the other species tested. Inhibition at such high concentrations may not be caused entirely by toxin, since the preparation was not completely pure. Nevertheless, our results indicate that resistant oats will tolerate > 400,000 times the concentrations of HV-toxin tolerated by susceptible oats. Thus, HV-toxin is much more selective than is HC-toxin.

Effects of methionine and sodium chloride on test plants.—Methionine was selected arbitrarily to compare inhibitory effects of a common nonspecific substance with the nonspecific inhibitory effects of high toxin concentrations. All seedling species listed in Table 3 were tested at 40, 400, and 4,000 μg methionine/ml. Root growth of radish, tomato, sorghum, and cucumber (the most sensitive plants tested) was inhibited 40% or more by methionine at 40 μg/ml, while corn, oats, and barley were < 20% inhibited. Methionine at 400 μg/ml gave 16-62% inhibition of plants listed in Table 3, while 4000 μg/ml gave 25-100% inhibition. Thus, plants vary somewhat in sensitivity to methionine. These results indicate that methionine is as inhibitory to plants in general as HV-toxin is to nonhost plants. However, the two substances may differ in dosage-response relations. For example, tomato was not affected by HV-toxin at 36 and 360 μg/ml, but was completely inhibited by 3600 μg (Table 4). Methionine, in contrast, gave 41, 58, and 100% inhibition of tomato root growth at 40, 400, and 4,000 μg/ml.

TABLE 4. Effect of *Helminthosporium victoriae* toxin on seedling root growth of host and nonhost plants

Plant	Growth of controls	% Inhibition at toxin concn (μg/ml) of:		
		36 μg	360 μg	3600 μg
	mm	%	%	%
Oat, cv. Park	50	100[a]	100	100
Oat, cv. Clinton	88	0	0	55
Tomato	49	0	0	100
Corn	100	0	0	80
Wheat	81	0	0	80
Sorghum	80	0	0	73
Barley	76	0	0	55

[a] Root growth of Park oats was completely inhibited by toxin at 0.009 μg/ml.

A comparison of methionine with HV- and HC-toxins on a molar basis may have little meaning because of instability of toxins. On a wt basis, HC-toxin had a much higher nonspecific inhibitory effect (Table 3) than either HV-toxin (Table 4) or methionine. The high degree of purity of some of the HC-toxin preparations, plus the inactivation data, indicate that contaminating substances do not account for all nonspecific toxicity.

The test plants listed in Table 3 also varied somewhat in sensitivity to sodium chloride. Root growth of tomato and radish was not affected by sodium chloride at 40, 400, and 4,000 μg/ml. The other test plants were inhibited 30 to 50% at 4,000 μg/ml. Many common substances may give nonspecific inhibition comparable to that of HV-toxin.

DISCUSSION.—Many studies have demonstrated the significance of HV-toxin as a determinant of pathogenicity (13, 18), but HC-toxin has had relatively little attention. The most convincing evidence that HC-toxin is required for pathogenicity comes from genetic tests (11) in which all hybrid progenies of *H. victoriae* × *H. carbonum* race-1 matings that produced HC-toxin were pathogenic to susceptible corn; all progenies that failed to produce HC-toxin were nonpathogenic. HC-toxin production was also correlated with pathogenicity in wild-type isolates of the fungus.

The correlation between relative toxin sensitivity and disease susceptibility in corn also supports the conclusion that HC-toxin is an essential determinant of pathogenicity. Corn inbreds and hybrids known from field data to be susceptible, resistant, or intermediate to *H. carbonum* had the same relative sensitivities to toxin. Similar correlations are known for *H. victoriae* and its toxin (16). The existence of plant types intermediate in toxin sensitivity suggests that there are quantitative differences in toxin receptors or transporting systems in resistant and susceptible cells.

Low levels of HC-toxin stimulated growth of susceptible seedling roots, and somewhat higher levels stimulated resistant roots. HC-toxin also stimulated incorporation of amino acids and uridine into trichloroacetic acid (TCA)-insoluble components of the cell (4), but higher concentrations and longer exposure times caused decreased growth and incorporations. By contrast, HV-toxin was never observed to stimulate growth, and very low levels quickly inhibited amino acid and uridine incorporation into TCA-insoluble components of the susceptible cell (13). High levels of HV-toxin had no effect on growth (Table 4) or incorporation by resistant plants (13).

Local stimulation of growth near the site of infection has been reported for several plant diseases (2, 14). This may be related to the stimulation of protein synthesis, as reported for several other plant diseases (1, 8), including a *Helminthosporium* infection of rice

plants (15). A comparative re-examination of all these phenomena would be desirable.

Hale et al. (3) previously described effects of *H. carbonum* culture filtrates that may have been caused by HC-toxin. Elongation of excised pea stems and susceptible corn coleoptiles was inhibited by diluted culture filtrates, while elongation of coleoptiles from *H. carbonum*-resistant corn was stimulated. We observed growth stimulation of some nonhost plants by purified HC-toxin at concentrations somewhat higher than those which stimulated susceptible corn. The basis of this growth stimulation is not known.

HC-toxin inhibited root growth of several nonhosts of *H. carbonum,* including radish, oats, and tomato. These plants were affected at lower concentrations of HC-toxin than were required to inhibit resistant corn, but tolerated higher levels than were tolerated by susceptible corn. The inhibitory effects on nonhost plants could be caused by impurities in the toxin preparations, but this seems unlikely because of careful preparation, data on inactivation, and high toxicity to susceptible corn. The effect of HC-toxin on some nonhost plants may not reflect activity on sites or mechanisms involved in disease development.

H. carbonum-resistant corn will tolerate approximately 100 times higher concentrations of HC-toxin than will *H. carbonum*-susceptible corn (10). This difference appears to be great enough to determine the fate of infection in the two different kinds of corn. The difference between *H. victoriae* resistant and susceptible oats in sensitivity to HV-toxin is much more striking; it is estimated from these and previous data (12) that resistant oats will tolerate at least 1,000,000 times higher concentrations than will susceptible oats. However, the effects of high toxin concentrations on resistant plants may involve different sites or mechanisms than those affected by low levels of toxin on susceptible plants. The better model to guide work on other diseases may be HC-toxin, because it may be similar to other determinants which lack the high degree of specificity shown by HV-toxin.

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