Leaf Bioassay for Helminthosporium carbonum Toxin – Search for Phytoalexin

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ABSTRACT

Resistance mechanisms in the Helminthosporium leaf spot disease of maize were investigated. Leaf extracts, obtained by boiling leaf-tissue homogenate or by centrifugation of intact leaf sections, were assayed for spore-germination inhibitory and mycelial-growth limiting substances. No such phytoalexin-like substances were found in extracts of inoculated leaves and no fungal nutrition deficiencies could be demonstrated. Applications of indole-3-acetic acid and *H. carbonum*-toxin influenced resistant host-pathogen interactions by increasing the size of lesions on resistant hosts, but did not overcome resistance. Double inoculation produced no cross-protection. A leaf bioassay for *H. carbonum*-toxin was developed which is ca. 10% as sensitive as the root bioassay but expresses a more meaningful relation to the disease symptoms. Differences in tolerance between susceptible and resistant maize leaves were demonstrated.

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Additional key words: host-specific toxin, fungistasis.

Helminthosporium leaf spot of maize (*Zea mays* L.), incited by *Helminthosporium carbonum* Ullstrup, is characterized by tan lesions, 1 to 20 mm in length, and round to oval in shape on leaves and ears of the susceptible host. If the host is resistant, pinpoint necrotic lesions appear at the site of fungal penetration, arresting further spread of the disease (5, 9).

Several inbred lines of maize are susceptible to the Helminthosporium leaf spot disease (e.g., lines Pr, K44, K61, 187-2) (11, 12).

The characteristic often used to identify the virulent race of *H. carbonum* (race 1) is the ability to produce a host-specific toxin (HC-toxin). A root-elongation bioassay (7, 10) was used to measure toxicity during procedures for isolation of HC-toxin.

A histological study (5) showed that spore germination and penetration (ca. 12 hr after inoculation) of host tissue by *H. carbonum* were equivalent on susceptible and resistant tissues. In susceptible tissue, hyphae branched freely and proliferated rapidly intracellularly, followed closely by breakdown of nuclei and chloroplasts, and collapse of cell walls. In resistant tissue, the infection hyphae usually branched once or twice but rarely filled the penetrated cell; most growth was confined to the initially penetrated epidermal cell. The fungus was not killed, however, since it was consistently isolated from the chlorotic flecks of resistant tissue, 60 days after inoculation.

Stimulatory effects of growth regulators such as indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) were reported (3) in the *H. carbonum*-maize interaction of susceptible maize line K44 and resistant line K41. Susceptible and resistant seedlings inoculated with *H. carbonum* race 1 showed an increase in leaf spots per plant and in average spot size. Inhibitory effects of IAA and NAA were shown on cultured fungus. In bioassay, the fungal culture filtrate inhibited pea stem sections and coleoptile sections of line K44. However, growth of coleoptile sections of line K41 was stimulated.

Stimulated root growth of susceptible and resistant maize seedlings by HC-toxin was reported (14) at concentrations well below toxic levels.

The cultivars used in this work were derived from lines Pr1, Pr, and K61. Lines Pr1 and Pr are nearly isogenic, the former being resistant to *H. carbonum* and the latter susceptible. Line K61 was used in hybrid form with Pr1 and Pr to obtain monohybrid expression of resistance (dominant) and susceptibility. Experiments were designed to indicate presence or absence of a phytoalexin and to study possible effects of HC-toxin on host-pathogen interactions in resistance-susceptibility phenomena. From these studies, a method of leaf bioassay was developed.

MATERIALS AND METHODS.–*Plant culture.* –Seeds of the hybrids, resistant (Pr1 × K61) and susceptible (Pr × K61) (referred to hereafter as Pr1 and Pr, respectively), were sterilized for 30 min by vacuum infiltration with 0.25% solution of hypochlorous acid containing a trace of Tween 80, followed by a rinse in running tap water for 12 hr. Plants used when less than 1 month old were grown in vermiculite-filled plastic pots (700 ml) under controlled conditions (day, 16 hr, 26 C; light intensity, 1200 ft-c [cool-white fluorescent-incandescent combination]), and were irrigated with tap water until seedling emergence and then with Hoagland's solution 1 (4), supplemented with 5 ppm iron chelate of ethylenediaminedi (*o*-hydroxyphenyl-acetic acid). Plants older than 1 month were grown in sterilized clay pots filled with a peat:sand:soil mixture (1:1:2, v/v) and watered with tap water, in a glasshouse at ca. 28 C. No difference

was noted between responses of plants from these two environments, neither of which had humidity control.

Inoculation.—Races 1 and 2 of *H. carbonum* were grown in the dark at 24 C on potato-dextrose agar for 14 days. Conidia were harvested in distilled water by gently stroking the mycelial mat with a stirring rod and straining through a fine nylon mesh. Spore density was adjusted to an OD of 0.45 (13 mm test tubes) at 375 nm with a Bausch and Lomb Spectronic 20 spectrophotometer. This spore suspension was sprayed on plants as a fine mist. Control plants received water spray. Sprayed plants were incubated in the dark 18 hr at 22 C, in a water-saturated atmosphere, before being returned to lighted conditions.

Toxin preparation.—HC-toxin from *H. carbonum* race 1 was prepared by a modification of the method of Pringle and Scheffer (10). The fungus was grown in still-culture for 28 days at 24 C in darkness using 2-liter Roux bottle and modified Fries No. 3 basal medium, supplemented with 0.1% Difco yeast extract. The culture liquid was filtered under vacuum and reduced to 5% of the original volume in 30 min in vacuo below 40 C. The concentrate was stored at 5 C for 48 hr, then filtered and added to an equal volume of methanol before storing at 5 C for 5 days and subsequent refiltering. The methanol was evaporated under vacuum below 35 C. The aqueous solution was extracted with chloroform until the extracts were colorless. The combined chloroform extracts were evaporated in vacuo to a thick brown syrup, diluted in 5 ml absolute ethanol, added to 100 ml diethyl ether, stored at 5 C for 1 week under nitrogen, and then filtered under nitrogen, using a glass-fiber filter. The light tan precipitate was stored under nitrogen in a desiccator at 5 C for possible further extractions of toxin. The filtrate was evaporated in vacuo to a very heavy syrup, which was extracted with small volumes of deionized water by storing for several days at 5 C, with occasional swirling of the mixture. The water extracts (two to six in number) were passed through a Bio-Gel P-2 column (100- to 200-mesh) and eluted with deionized water. The toxin-containing fractions, as determined by high conductivity measurements (6) were pooled and concentrated in vacuo below 35 C. This clear, colorless concentrate was stored at 5 C.

HC-toxin bioassay on leaves.—The following leaf bioassay was developed for HC-toxin. Randomly selected leaves of maize plants older than 1 month were inverted and gently mounted on Plexiglas plates so that no physical damage occurred to the leaves. The middle portions of the inverted leaves were held against the plates with glass rods, exposing a 10-cm-long section of the leaf underside in a horizontal position. This section was prepared by lightly rubbing it with dry Carborundum powder, later removed with moist tissue paper. This treatment aided the wetting of the leaf with test solution and produced consistent results; no visible reaction occurred in water-treated control plants.

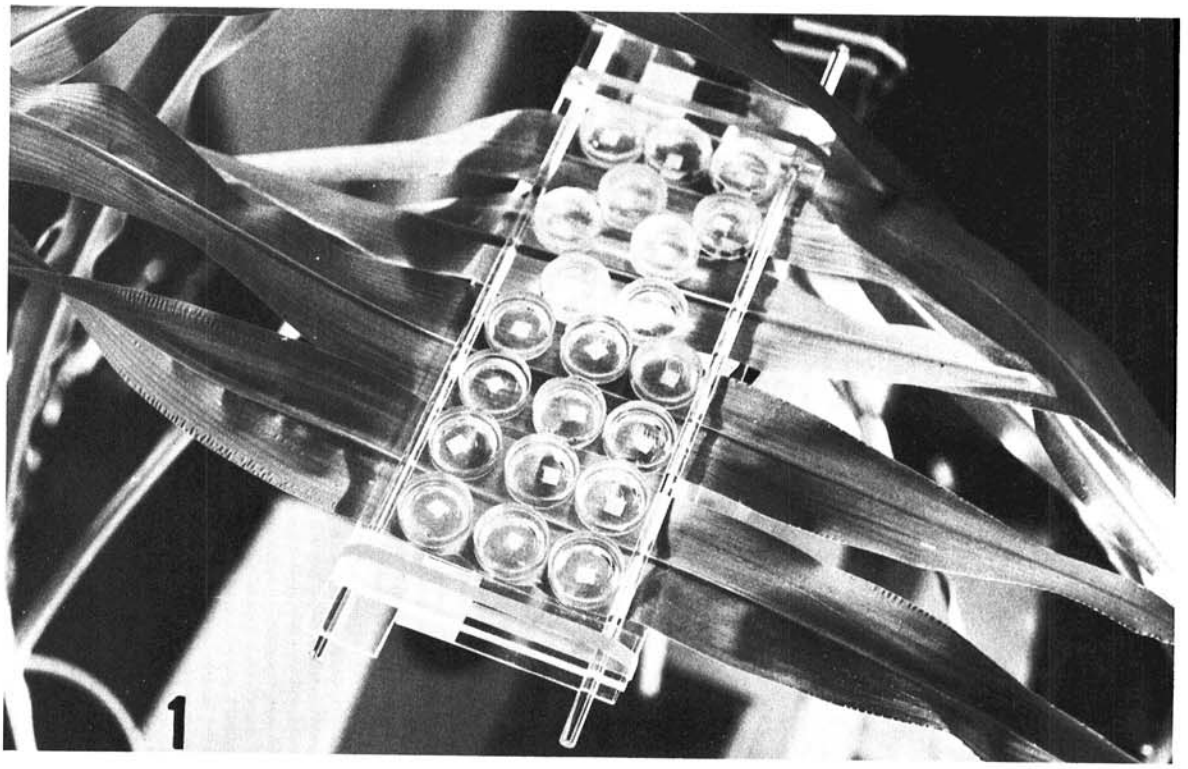
Small pieces of Beckman electrophoresis wick paper (5 × 5 mm) were placed on the treated areas. Test solutions were pipetted onto the wicks (50 μliter/wick) before covering them with inverted glass planchets to minimize evaporation (Fig. 1). Where evaporation occurred due to wrinkles in the leaf margin, the wicks were periodically watered with deionized water.

The 1-month age of the plants was chosen because the leaves are generally wide enough to accommodate a glass planchet between the midrib and the leaf margin. For much younger plants, an area of the underside of a leaf was treated with Carborundum. The wick paper squares were placed on the Plexiglas plate. The leaf was placed on the wicks so that the treated areas of the leaf blade covered the paper wicks. Glass microscope slides were placed across the leaf and along its sides. This held the leaf gently on the wick squares, and retarded evaporation. Lesion development was observable after 2 to 7 days (Fig. 2).

Search for phytoalexin by spore germination.—Techniques which proved successful in the case of *H. turcicum* (8) were used to search for a phytoalexin-like substance induced by *H. carbonum*. Four-week-old maize plants, Pr1 and Pr, were inoculated (10 ml each) separately with races 1 and 2 of *H. carbonum*. After 8 days, leaves were severed (mid-veins excised) and cut into small segments. Five g of segments in 50 ml water was macerated in a Virtis homogenizer for 5 min, refluxed 1 hr, stored at 5 C for 24 hr and filtered. The filtrate was concentrated to 5 ml under vacuum below 40 C. One drop of spore suspension was mixed with two drops of each concentrate on a microscope cavity-slide and placed in a high-humidity dark chamber for 8 hr.

In a modification of the technique, leaf tissue segments were cut into 5-cm-long sections and placed in perforated polyethylene centrifuge tubes, which were in turn inserted into larger centrifugation bottles half-filled with glass beads. After centrifugation at 8,500 *g* for 20 min, the liquid collected between the

Fig. 1-5. 1) Apparatus for mounting leaves in bioassay. Inverted leaves are held in place by glass rods. Inverted glass planchets cover the square wicks containing the test solutions. — **2-3)** Leaves of 10-week-old maize plants treated with *Helminthosporium carbonum*-toxin solutions, from left to right: 240, 120, 48, and 12 μg/ml. Small dark squares with heavy lines through them are test wicks held in place by tape only during photography. Each of the six wicks per leaf contained 50 μliters of the same concentration of toxin. Wick size is ca. 5 × 5 mm. **(2)** Susceptible (Pr)—lesions were seen at 48 μ/ml. Large dark squares at lower edge of figure are labels. **(3)** Resistant (Pr1)—No lesions were observed—**4-5)** Leaves of 7-week-old maize plants treated with *Helminthosporium carbonum*-toxin solutions, from left to right: 20, 39, 78, 125, 156, 250, 312, 625, and 1,250 μ/ml. **(4)** Susceptible (Pr)—faint lesions were visible at the lowest concentrations. **(5)** Resistant (Pr1)—faint lesions were visible at 312 μg/ml.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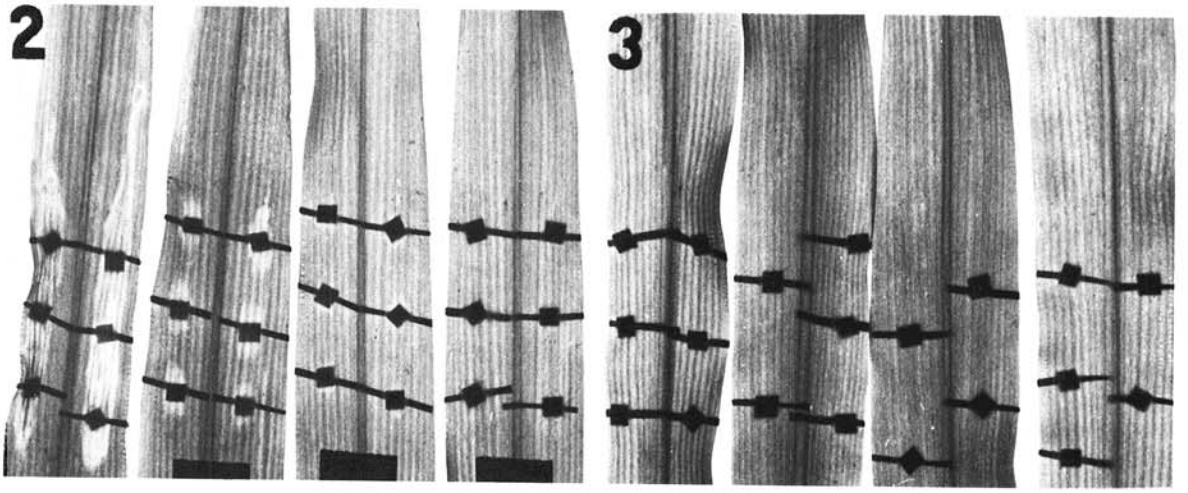
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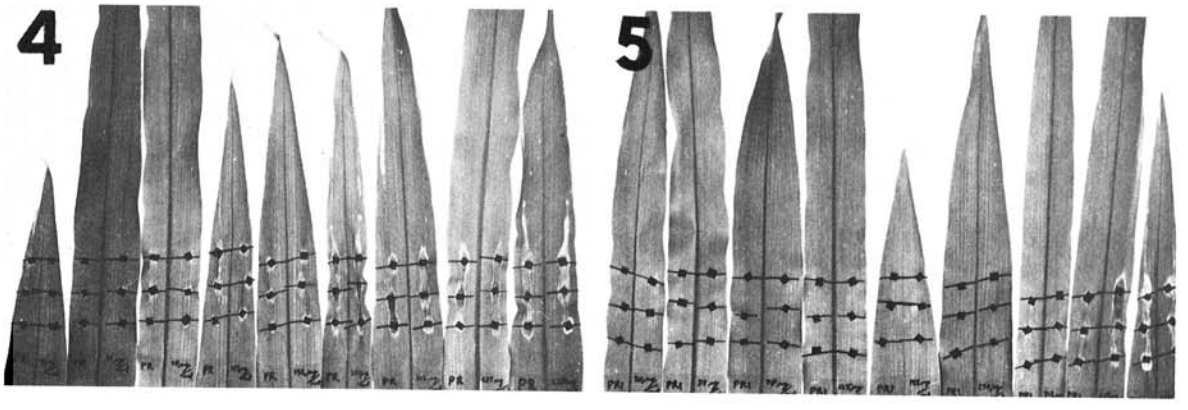
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glass beads represented 5% of the fresh tissue weight. This liquid was sterilized by filtration through 0.22-μ Millipore filters and assayed.

Leaf sections were also vacuum-infiltrated with water before centrifugation. The liquid collected represented 10% of the fresh tissue weight.

Limitations of fungal growth.—Method a) To detect limitations of fungal growth in later stages, aliquots of the liquid obtained from centrifugation of water-infiltrated tissue were distributed into 100-ml Erlenmeyer flasks, 5 ml per flask. These flasks were inoculated with 0.5 ml of spore suspension and stored in the dark at room temperature. After 4 weeks, the growth and color of the cultures were assessed. Mycelial growth of *H. carbonum* is whitish-gray, while conidia in aggregate are black to black-brown.

Method b) Six-week-old Pr1 and Pr plants were inoculated separately with races 1 and 2 of *H. carbonum*, using 30 ml of spore suspension per plant. Two weeks after inoculation, extracts of these leaf tissues were made using water-vacuum infiltration before centrifugation. Extracts were diluted with an equal amount of water and sterilized by filtration. Four ml of each, in 50 ml Erlenmeyer flasks, were inoculated with minute pieces of *H. carbonum* cultures (dry weight 4×10^{-4} g or less) grown on potato-dextrose agar. Cultures were grown in the dark at room temperature.

Three days after inoculation, mycelial mats covered the liquid surface in the flasks. Cultures were filtered and washed on tared filter paper, dried at 70 C for 3 days, and weighed.

Cross-protection.—Seedlings of Pr1 and Pr were inoculated with races 1 or 2. After 4 days, a second inoculation with the other race was performed in the same manner, resulting in all possible combinations of hosts and inoculations.

Effects of HC-toxin and IAA on resistance.—Two-week-old Pr1 and Pr plants were inoculated separately with races 1 and 2 of *H. carbonum*. Two days later, randomly selected leaves were mounted horizontally for leaf bioassay but the leaves were not treated with Carborundum. Wicks, containing toxin or 10^{-5} M IAA solution, were placed in contact with the inoculated leaves. Toxin concentrations were just below those which produced symptoms of toxicity in leaf bioassay. Twelve days after inoculation, sizes of the lesions were measured under a dissection microscope by measuring the diameters (to nearest 0.1 mm) and calculating the area, using the formula for area of an elipse.

In a variation of this technique, instead of spraying inoculum in a fine mist onto the leaf surface, the inoculum was pipetted onto strips of wick paper (0.35 ml inoculum on a 53- x 5-mm strip), and the wick was applied to the leaf surface treated with Carborundum. The usual dark treatment was omitted, due to the bulkiness of the leaf-mounting apparatus, but high humidity was assured by the moist wick in contact with the leaf. After 24 hr, the inoculation wicks were replaced by wicks containing the treatments listed in Table 1 (control plants treated with water).

RESULTS.—*Phytoalexin and fungal growth.*—Spore germination was over 95% in extracts of inoculated or noninoculated tissue in all cases and with both races. Hyphal proliferation appeared significantly higher in extracts than in water controls.

The sizes of mycelial mats of both races of *H. carbonum* grown on inoculated and noninoculated, resistant and susceptible, tissue extracts were equivalent. The quantities of mycelia were difficult to assess, because many small fluffy colonies had formed due to inoculation with a spore suspension. However, differences in degrees of black color development (indicating spore formation) occurred in healthy maize-tissue extracts. Extracts of noninoculated Pr produced fewer spores of race 1 and more spores of race 2 than did all other extract-*H. carbonum* combinations. Spore formation in inoculated tissue extract-*H. carbonum* combinations appeared equivalent to that in healthy Pr1 tissue extract-*H. carbonum* combinations.

Weights of colonies, for which the inoculum was a

TABLE 1. Treatments of HC-toxin or indole-3-acetic acid on inoculated maize leaves prepared with Carborundum

Treatments	Host-pathogen combinations[c]			
	Pr		Pr1	
	Race 1	Race 2	Race 1	Race 2
Toxin conc. #1 (μg/ml)[a]	208	208	625	625
Toxin conc. #2 (μg/ml)[b]	21	21	208	208
Indole-3-acetic acid	10^{-5} M	10^{-5} M	10^{-5} M	10^{-5} M

[a]Toxin concentration #1 will produce symptoms of toxicity alone.

[b]Toxin concentration #2 will not produce symptoms alone.

[c]Pr and Pr1 are susceptible and resistant, respectively.

TABLE 2. Dry weights of 3-day-old mycelial pads of *Helminthosporium carbonum*, cultured on diluted extracts from healthy and inoculated Pr1 and Pr maize leaves[b]

Source of extract	Weights of *H. carbonum* cultures (mg)[a]	
	Race 1	Race 2
Pr1 – noninoculated	11	12
Pr1 – inoculated race 1	17	17
Pr1 – inoculated race 2	20	9
Pr – noninoculated	23	17
Pr – inoculated race 1	5	7
Pr – inoculated race 2	15	19

[a]Each weight is the average of two cultures.

[b]Pr and Pr1 are susceptible and resistant, respectively.

piece of culture on agar, varied from different extract-inoculum combinations (Table 2); however, the colonies appeared uniform in size and spore formation was equally good in all cases.

Thus, we conclude that no phytoalexin-like substance was evident in the extracts.

Cross-protection.—Five days after the second inoculation of 5-week-old seedlings, symptoms of all reactions were well expressed. Symptoms of the second inoculation were not visibly influenced by the first inoculation. All gradations of proximity between necrotic spots of the two inoculations were evident. Where the second inoculation produced a susceptible reaction, the rapidly spreading lesions engulfed the necrotic spots of the resistant first-inoculation reaction. In susceptible reactions, where isolated lesions occurred, perhaps produced by single spores or spores aggregated in a small clump, the spreading lesion was surrounded by a halo of chlorotic tissue. This halo preceded the spreading lesions. No such halo was observed in the resistant reactions. The halo may be the result of HC-toxin action in susceptible tissue.

Repetition on 3-week-old susceptible plants gave similar results. The halo effect was less distinct; instead relatively large areas rapidly became chlorotic.

No evidence for a phytoalexin-like substance was found. Although these observations do not rule out the existence of such a material in the resistant *H. carbonum*-host interaction, it seems unlikely that a phytoalexin is a controlling factor in the resistant reactions. More probably necrotic areas present a barrier to further intracellular invasion by the growing fungus.

Effects of HC-toxin and IAA on resistance.—Table 3 indicates that toxin, in concentrations below the threshold of lesion induction, or IAA increases the size of necrotic lesions, but does not completely overcome the resistance mechanisms. Further increase in size of lesions by higher toxin concentrations would be the result of toxin action alone.

TABLE 3. Effects of indole-3-acetic acid and *Helminthosporium carbonum* toxin on size of lesions in resistant reactions of maize to *H. carbonum* inoculations

Treatment	Lesion areas (mm^2) of maize-*H. carbonum* combinations[a]		
	Pr	Pr1	
	Race 2	Race 2	Race 1
H_2O control	0.35	0.35	0.20
HC-toxin[b]	0.99	0.55	0.70
IAA (1×10^{-5} M)	1.31	0.93	1.16

[a]Areas of lesions represent averages of at least six leaf areas of 1 cm^2 each, 12 days after inoculation by spray. Pr and Pr1 are susceptible and resistant, respectively.

[b]Toxin concentrations 25 and 250 μg/ml for Pr and Pr1, respectively.

Necrotic lesions develop on leaf tissue if sufficient toxin is applied to the leaf surface. The presence or absence of a lesion was used as the criterion of toxicity, since the lengths or areas of the lesions were generally in proportion to concentration only when higher toxin concentrations were used. Typical results of the toxin bioassay are shown in Fig. 2 and 3. Toxin concentrations between 20 and 30 μg/ml produced lesions on susceptible hosts. Concentrations of 300 to 400 μg/ml were required for resistant leaves. Symptoms are well developed 72 hr after application, but are visible sometime before that, depending on the age of the tissue and on the toxin concentration used. No lesion induction occurred on plants treated with water-containing wicks.

HC-toxin is toxic to resistant maize root tissue at concentrations ca. 100 times higher than that for susceptible tissue (7, 10). The toxin concentration needed to induce lesions in resistant leaf tissue is ca. 10 times higher then that required for susceptible leaf tissue (Fig. 4 and 5).

DISCUSSION.—Production of a host-specific toxin by the fungus undoubtedly plays the primary role in the success of attempted colonization on the host, depending on whether or not the host is sensitive to that toxin. However, the findings that the non-toxin-producing race 2 of *H. carbonum* successfuly parasitizes the ears of susceptible maize, while most other parts of the plant are resistant (9, 12, 13), points to other resistance mechanisms apart from tolerance to the HC-toxin.

Attempts to isolate a phytoalexin-like substance like the one induced in maize by *H. turcicum* (8) failed. Instead, both races of *H. carbonum* grew luxuriantly on extracts from inoculated maize leaves. In a nutrition study of *H. carbonum* race 1 (2), alcohol extracts of inoculated susceptible maize leaves of line K44 retarded growth of race 1. In the present work, similar findings were made for non-alcoholic extracts of inoculated susceptible leaves of Pr. In addition to retardation of mycelial growth of races 1 and 2 by extracts of race 1-inoculated susceptible leaves, mycelial growth of race 2 was also retarded by extracts of race 2-inoculated resistant leaves (Table 2). However, inhibited colonies appeared normal, and therefore such inhibiton may not indicate the presence of fungistatic compounds, but possibly altered carbon or nitrogen sources, changed pH, or other phenomena.

Successful colonization on ears of susceptible Pr by nontoxin-producing race 2 of *H. carbonum* also may be due to nutritional phenomena. In field-grown leaves of another susceptible line of maize, K44, the sugar content was more than twice that of the resistant line, K41 (2). In this same study, growth of race 1 increased with increasing sugar concentrations. If such considerations as higher sugar content in susceptible tissue also hold true for inbred line Pr, and if race 2 responds to higher sugar concentrations as does race 1, then successful colonization on the ears of Pr may be due to the improved nutrition which race 2 encounters on these ears, with presence

or absence of fungistatic compounds being questionable.

Since attempts to extract fungistatic substances were unsuccessful, we investigated expressions of such substances in vivo. If inhibition of fungal growth through an induced substance is responsible for one or more of the resistant reactions, it is likely that the induced substance is active near the site of fungal arrest. In the search for such a substance by investigation of cross-protection by double inoculation, no influence of the first inoculation upon the second inoculation was found. Instead, in cases where the second inoculation of susceptible host (previously inoculated with non-toxin-producing race 2) was with the toxin-producing race 1, chlorotic areas spread through the leaf tissue, engulfing all previous lesions. This chlorosis is probably due to toxin action which permeates the tissue in the wake of necrosis. In younger plants, this necrosis appears so rapidly that large tan lesions, characteristic of older tissues infected with race 1 of *H. carbonum*, never appear. Instead, the leaves showed relatively small, race-1 lesions, and turned dark green to black in color as they became dry and crisp. It was noted, also, that in the chlorotic areas where lesions of race 2 were established, no increase in size of the race-2 lesions was observed. If race 2 remains alive in resistant lesions (5) under these conditions, presence of HC-toxin from the neighboring growth of race 1 seems inadequate to overcome the resistance to race 2.

In earlier work HC-toxin was added to the inoculum of *H. victoriae*, which is not pathogenic to maize, with the effect that limited but abnormal growth of *H. victoriae* occurred on maize (1). IAA, applied to other cultivars of maize plants, resulted in increased size and number of lesions (3). The present work also shows increased size of lesions for the resistant reactions of Pr and Prl to *H. carbonum* when HC-toxin or IAA were applied to the leaf tissue. This also showed that the resistance mechanisms were not overcome, and it appears that for successful virulence of *H. carbonum* on maize, toxin production by the fungus, as well as susceptible tissues, is necessary. The virulence of race 2 on ears but not on leaves of susceptible Pr host is still an enigma.

Development of the leaf bioassay aided observations and assessments of the HC-toxin in tissues where the host-pathogen interactions normally occur, making results biologically more related to disease symptoms. This assay is less sensitive than the root elongation bioassay, since no stimulation of leaf tissues is evident for low toxin concentrations, whereas in the root elongation bioassay, stimulation of root elongation is observed. In the root elongation bioassay, potency of an HC-toxin preparation is usually expressed in terms of the toxin concentration needed to cause 50% inhibition of root elongation, while in the leaf bioassay, potency is expressed in terms of the toxin concentration needed for lesion formation. Leaf tissues appear less sensitive to HC-toxin than elongating roots. The concentration needed for lesion formation is estimated as 10 times higher than that needed to cause 50% inhibition of root elongation.

A modification of the leaf bioassay facilitated use of leaves from young seedlings. A disadvantage of using young leaves is that if a sizeable necrotic lesion is produced, it is likely to affect the water supply for regions between lesion and the leaf tip, causing wilting and arresting further development of the lesion. In application of toxin concentrations which are moderately to excessively toxic, young leaves may turn dark green to black in color and become dry and crisp before sizeable lesions can develop. However, this does not interfere with the bioassay if one considers this difference in symptom expression.

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