Resistance in Susceptible Maize to *Helminthosporium carbonum* Race 1 Induced by Prior Inoculation with Race 2

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ABSTRACT

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Inoculation of leaves of susceptible maize genotypes with the nonpathogenic race 2 of *Helminthosporium carbonum* at least 10 hr before inoculation with the pathogenic race 1 prevented development of large lesions typical of the susceptible reaction. Appressorium formation, penetration, and hyphal growth by the pathogen were decreased. Addition of HC-toxin (the host-specific toxin produced by pathogenic race 1) to the race 1 challenge inoculum abolished the resistance induced by race 2. Inoculation with *H. victoriae*, *H. turcicum*, or *Alternaria* sp. at least 18 hr before inoculation with *H. carbonum* race 1 also induced resistance. The results suggest that a general resistance mechanism is activated upon contact of the maize leaf with a potential pathogen and that HC-toxin plays a role in pathogenesis by preventing or overcoming those events.

Additional keyword: cross-protection.

The fungus *Helminthosporium carbonum* Ullstrup (syn. *Bipolaris zeicola* (G. L. Stout) Shoemaker) race 1 causes a leaf spot on maize (*Zea mays* L.) (20,36) and produces a host-specific toxin (HC-toxin) that is the determinant of pathogenicity and host specificity (26,28,30,31). Race 2 isolates of this fungus do not produce toxin and are not pathogenic, although they can penetrate leaves and elicit small necrotic lesions. Maize genotypes resistant to the pathogen are insensitive to HC-toxin, whereas susceptible genotypes are sensitive. The mechanism of action of HC-toxin and its precise role in pathogenesis are not known. However, when HC-toxin is added to the conidial inoculum of *H. carbonum* race 2 or to *H. victoriae* F. Meehan & Murphy, an oat pathogen, the result is a susceptible reaction (6) characterized by extensive colonization of leaf tissue. Thus, HC-toxin either prevents the general resistance mechanisms in toxin-sensitive tissue that restricts colonization by potential pathogens (6,29), or it induces specific physiological and biochemical responses that result in susceptibility (7,22).

In a number of plant species, preliminary inoculation of a susceptible host with a nonpathogenic organism induces resistance or protection to one that is normally pathogenic (15,22). Induced resistance often is nonspecific, is induced by bacteria, fungi, or viruses, individually or in combination, and can be localized or systemic depending on the host-pathogen combination (22). For example, resistance of green bean to the anthracnose pathogen, *Collectotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., can be induced by inoculation with a nonpathogenic isolate of that fungus or with *Alternaria* species or *H. carbonum* (27). The host response is systemic, and complete protection against a challenge inoculation is effective at a distance from the inducing inoculation site (10). Likewise, protection in cucumber (8,9) and tobacco (35) to *C. lagenarium* (Pass.) Ellis & Halst and *Peronospora tabacina* D. B. Adam, respectively, also is systemic and is graft-transmissible (13). On the other hand, resistance to infection by *Erysiphe graminis* DC., induced in leaves of barley when inoculated with an incompatible race, is localized at the site of preliminary inoculation (23–25). This resistance is expressed as a decrease in the infection frequency and the length of secondary hyphae. Viral-induced resistance in tobacco is effective against challenge by fungi as well as other viruses (1,18,19). In addition, tobacco plants can be protected against *Pseudomonas solanacearum* by the injection of dead cells or with the lipopolysaccharide from membranes of several gram-negative bacteria (33). Despite the relatively common occurrence of this phenomenon, no evidence for induced resistance in maize to *H. carbonum* has been observed by previous investigators (6,12).

Our study demonstrates that inoculation of susceptible maize leaves by *H. carbonum* race 2 induces resistance to the pathogenic race 1. The resistance is manifest as a decrease in appressorium formation and penetration of leaf tissue by conidia of race 1 and consequently results in resistant-type lesions. Addition of HC-toxin to the conidial suspensions abolishes the protection, suggesting that the toxin plays a role in pathogenesis by preventing or counteracting the mechanisms responsible for induced resistance.

MATERIALS AND METHODS

Fungi and plant material. The genotype of maize used in this study was the hybrid K61 × Pr (susceptible to *H. carbonum*). Plants (5–10 per 20-cm pot) were grown and maintained in the greenhouse at 23–30 C and supplemented with 16 hr of fluorescent lighting.

The wild-type races 1 and 2 and an albino mutant of race 1 (obtained from Dr. R. L. Nicholson, Purdue University) of *H. carbonum* were isolated from infected leaves and stored on silica gel (34). These fungi were grown at 26 C on lactose casein hydrolysate (LCH) medium (34). Cultures of *H. turcicum* race 1, *H. victoriae*, and *Alternaria* were grown at 26 C on either LCH or potato-dextrose agar (Difco).

Inoculations. Conidia from 2- to 3-wk-old cultures of the various fungi were harvested in 0.01% (v/v) aqueous Tween 20. Concentrations of conidia were determined with a hemacytometer and adjusted to either 5×10^3 or 1×10^4 conidia/ml.

The fourth to seventh leaves of 3- to 4-wk-old plants were inoculated in microhumidity chambers (3). Each chamber consisted of two pieces of clear acrylic enclosing the leaf and a layer of foam padding. They were held together with one-inch binder clips and supported by a ring stand. Inoculum/treatments were applied in 25-μl volumes through wells (6 mm diameter) in the top half of the chamber. Wells were then covered with clear cellophane tape to prevent evaporation of the fluids.

HC-toxin, purified as described by Ciuffetti et al (5) or generously supplied by Dr. V. Macko (Boyce Thompson Institute, Ithaca, NY), was used in challenge inoculations to examine its effect on the induced resistance.

Microscopic observations. Detached leaf sections (4–5 cm × 1.5–2 cm) from susceptible plants were placed in petri dishes containing moist filter paper. Leaf pieces were inoculated with four 15-μl droplets of conidial suspensions (5×10^3/ml) of *H. carbonum* race 1 or race 2 and incubated at 25 C. After defined intervals, leaf pieces were removed, cut into individual squares, and placed directly into lactophenol/cotton blue (0.05%) for germination counts or into a clearing solution of ethanol/acetic acid (3:1, v:v) for at least 24 hr. Leaf pieces used to quantify appressoria and penetration sites were removed from the clearing solution and placed in a 0.01 M potassium phosphate buffer, pH 7.4, for at least 10 min and then stained under vacuum for about 2 hr with lactophenol/cotton blue (0.05%).

RESULTS

Induced resistance. Inoculation of susceptible leaves with race 2 of *H. carbonum* induced resistance to challenge inoculation with race 1. Leaves were inoculated with conidia of race 2 for 12 hr, and then the inoculum was removed and replaced (challenged) with race 1 conidia for an additional 12 hr. Lesions on challenged tissue were substantially reduced in size and in number compared to those produced by unchallenged race 1 inoculations (Fig. 1). Preinoculation with race 2 changed the normal susceptible response to that of a race 2, resistant-type lesion. However, when tissue was preinoculated with race 2 and then challenged with race 1 conidia supplemented with HC-toxin (5 μg/ml; 125 ng/well), the induced resistance was nullified, and a susceptible-type lesion was observed similar to that produced by inoculation with race 1 alone or race 2 conidia plus HC-toxin (Fig. 1). Simultaneous inoculation with conidia of races 1 and 2 or inoculation with race 1 followed by race 2 did not alter the susceptible reaction (data not shown).

Conidia of race 2 induced resistance only when they preceded inoculation with race 1 conidia by at least 10 hr (Fig. 2). Susceptible-type lesions formed if race 2 inoculum was removed before that time. Leaves of the susceptible genotype were protected against infection by race 1 when the concentration of the race 2 conidia was greater than 2,500 to 5,000 conidia/ml or about 2.2–4.4 conidia/mm^2 (data not shown).

Other fungal species also induced resistance. *H. victoriae, H. turcicum,* and an *Alternaria* sp. all protected against challenge inoculation with *H. carbonum* race 1, but only when inoculation with the inducer preceded inoculation with the challenger by 18 hr (Fig. 3). However, lesions were slightly smaller when the race 1 challenge followed inoculation with conidia of *H. victoriae* by only 12 hr (Fig. 3).

Microscopic observations of infection by *H. carbonum*. The establishment of the albino mutant of race 1 in susceptible maize tissue was reduced after preinoculation with the wild-type race 2 (Fig. 4). The albino mutant, which penetrated and colonized leaf tissue similarly to the wild-type of race 1, was used because its hyphae could be distinguished from the initial race 2 inoculum. Conidia of challenge race 1 and race 1 alone (no preinoculation with race 2) germinated at nearly the same rate and to the same extent (Fig. 4, inset). However, challenge race 1 had fewer appressoria and penetrations compared with the race 1 control. Also, microscopic observations showed that mycelial development of

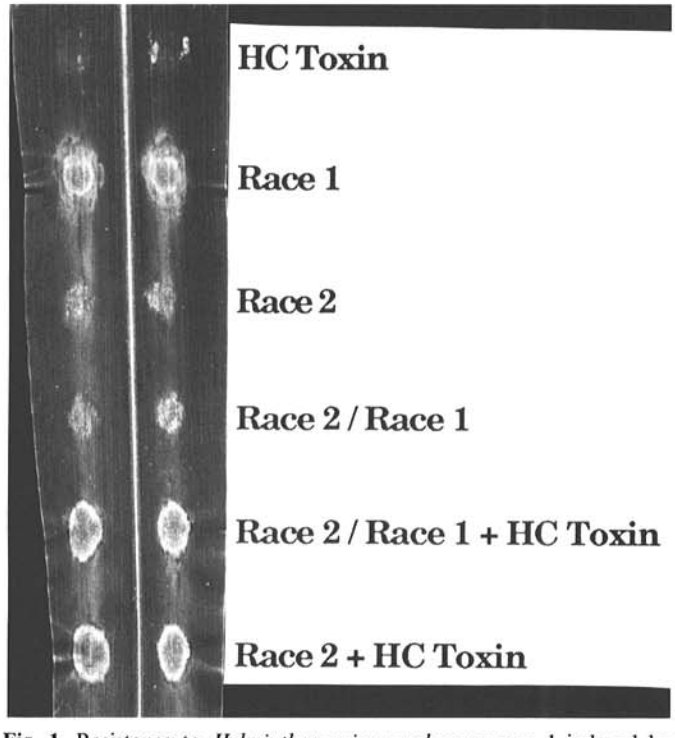

Fig. 1. Resistance to *Helminthosporium carbonum* race 1 induced by race 2 on leaves of the susceptible hybrid K61 × Pr. Leaves in microhumidity chambers were inoculated with conidia of race 1 alone or race 2 alone or in the presence of HC-toxin (5 μg/ml) for 24 hr or preinoculated with race 2 for 12 hr and then challenge-inoculated for an additional 12 hr with conidia of race 1 or race 1 plus HC-toxin. The inocula were removed 24 hr after the first inoculation, and the photo was taken 3 days later.

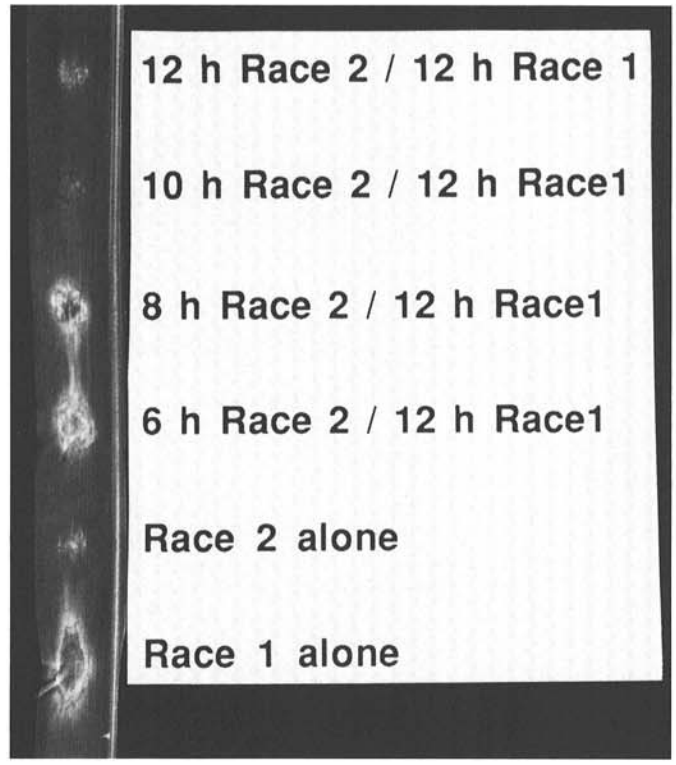

Fig. 2. Time of preinoculation with *Helminthosporium carbonum* race 2 required to induce resistance to race 1 on leaves of the susceptible hybrid K61 × Pr. Leaves in microhumidity chambers were inoculated with conidia of race 2 for 6, 8, 10, or 12 hr and challenge-inoculated with conidia of race 1 for an additional 12 hr when the chambers were removed. The photo was taken 4 days after inoculation.

the challenge fungus on the leaf surface was sparse compared to the abundant growth of control race 1 (data not shown).

Addition of HC-toxin to the challenge inoculum prevented the induced resistance. Susceptible leaves were preinoculated with race 2 and challenged with race 1 or race 1 plus HC-toxin (Fig. 5). Rates of conidial germination in both types of challenge treatments were very similar, as they were in the previous experiment (Figs. 4 and 5). Appressorium formation and penetration by race 1 and race 1 plus HC-toxin were virtually identical for the first 12 hr. However, with increased time of incubation, the number of appressoria formed by race 1 plus HC-toxin, and their subsequent penetration increased significantly compared with challenge race 1 in the absence of toxin. When these leaf lesions were incubated in a moist chamber, conidia of albino race and wild-type race 2 were produced, indicating further that treatment with HC-toxin assisted pathogenesis by race 1 and did not merely enhance growth of race 2.

DISCUSSION

Induced resistance has been well documented in a variety of host-pathogen systems (15,22). Inoculation of a susceptible plant with a nonpathogenic isolate results in the induced resistance or cross-protection of the infected tissue to a normally pathogenic isolate. This protection may be either localized or systemic. In our study, inoculation of susceptible leaves with the nonpathogen, *H. carbonum* race 2, 10–12 hr before the challenge inoculation with race 1 resulted in restricted, necrotic lesions instead of expanding, susceptible-type lesions.

Our findings at first appear to conflict with results of previous studies (6,12). Comstock and Scheffer (6) inoculated susceptible maize leaves with the oat pathogen, *H. victoriae*, and then challenge inoculated the leaves 24 or 48 hr later with *H. carbonum* race 1. *H. carbonum* developed equally well in controls and in previously inoculated tissue. In another study, Hoffman and Zscheile (12) observed a susceptible reaction when race 1 of *H. carbonum* was inoculated 4 days after the first inoculation with race 2. However, neither study specified the location of the challenge inoculation. We found that the induced resistance is effective over a localized area and that a minimum density of 2.2–4.4 conidia of race 2/mm^2 is necessary to induce resistance. Although the number of conidia used in the previous experiments (6,12) was apparently sufficient to induce protection, the distance separating penetration sites from the first and second inoculations may have been enough to avoid or circumvent the resistance mechanism(s).

The induced resistance was evident as a reduction in the number of appressoria, their penetration into leaf tissue, and subsequent development of resistant-type lesions. Because the growth and development of race 1 in the host tissue was significantly restricted, it was unable to overcome the resistance mechanism induced in leaves preinoculated with race 2 conidia unless HC-toxin was provided with the challenge inoculum (Fig. 1). These results suggest that insufficient amounts of race 1 were present to produce and release adequate quantities of HC-toxin to suppress or overcome the resistance mechanism.

These observations illustrate the complexity and dynamic nature of the host-pathogen interaction. The host response may

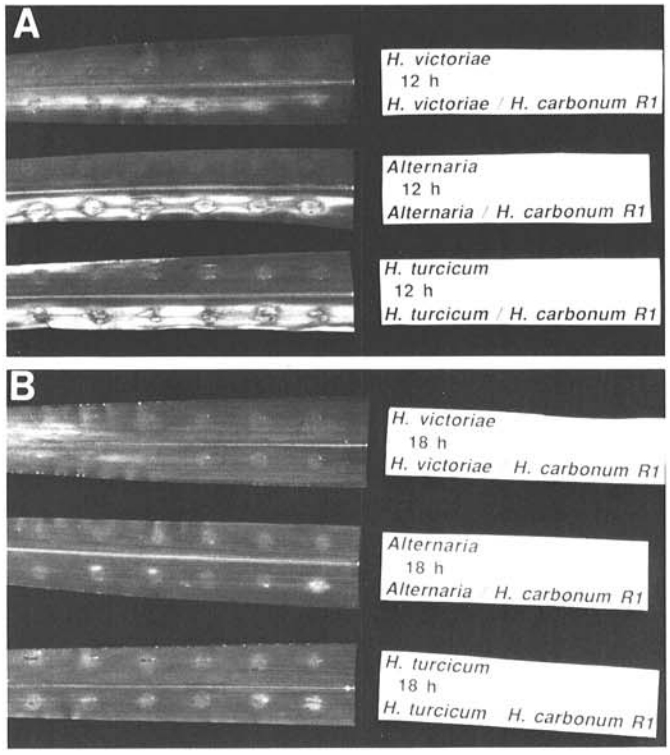

Fig. 3. Resistance to *Helminthosporium carbonum* race 1 induced by prior inoculation with *H. victoriae, H. turcicum,* or *Alternaria* on leaves of the susceptible hybrid K61 × Pr. Leaves in microhumidity chambers were inoculated with the inducer for **A,** 12 hr or, **B,** 18 hr and then challenged-inoculated with race 1 for an additional 12 hr when the chambers were removed. The photo was taken 5 days after inoculation.

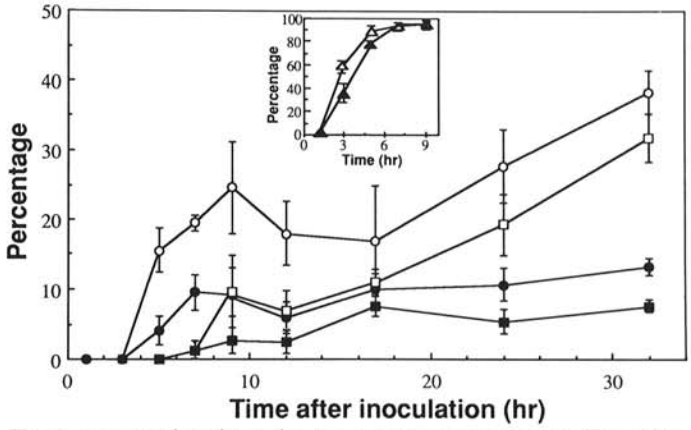

Fig. 4. Appressorium formation (○, ●) and penetration (□, ■) by the albino mutant of *Helminthosporium carbonum* race 1 on leaves of the susceptible hybrid K61 × Pr. Leaves were inoculated with conidia of the albino mutant of race 1 (open symbols) or preinoculated with conidia of wild-type race 2 for 12 hr and then with the albino mutant of race 1 (closed symbols). Inset shows germination of control and challenge race 1 conidia (△, ▲). Data for appressorium formation and penetration are expressed as the percentage of germinated conidia. Bars represent standard errors of three replicates.

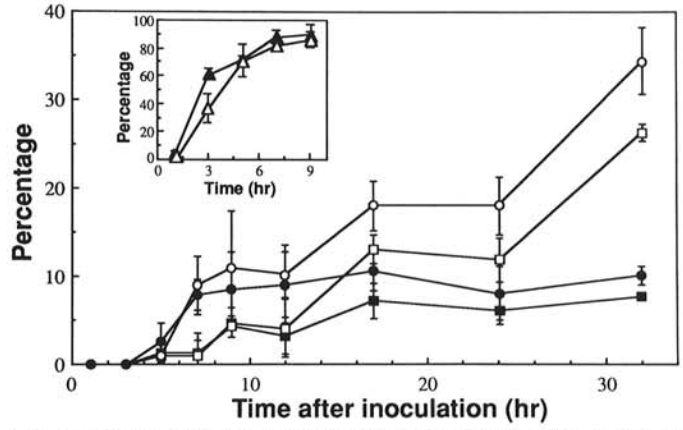

Fig. 5. Appressorium formation (●, ○) and penetration (■, □) by the albino mutant of *Helminthosporium carbonum* race 1 on leaves of the susceptible hybrid K61 × Pr. Leaves were preinoculated with conidia of wild-type race 2 for 12 hr and then with the albino mutant of race 1 in the absence (closed symbols) or presence (open symbols) of HC-toxin (5 μg/ml). Inset shows germination of challenge race 1 conidia in the absence and presence of HC-toxin (▲, △). Data for appressorium formation and penetration are expressed as the percentage of germinated conidia. Bars represent standard errors of three replicates.

be enhanced or suppressed by quantitative and qualitative effects on the recognition events in the host that are induced directly by processes of the pathogen (i.e., germination, formation of infection structures, penetration), by specific disease determinants (i.e., host-specific toxins), or by an individual component of the host itself. In powdery mildew of barley, the primary recognition of the pathogen, *E. graminis*, by the host cells becomes irreversible once the cells are physiologically conditioned (23–25). However, in the present study, addition of HC-toxin to challenge race 1 inoculum reversed or suppressed the expression of induced resistance in maize leaves. Similarly, toxin released by conidia of *A. alternata* before host invasion suppresses the resistance induced by fungi nonpathogenic to pear (11,14).

A response of the host appears to be required for resistance to be induced. This is supported by the observations that a period of at least 10 hr is required for protection and that other nonpathogens (e.g., *H. victoriae*, *H. turcicum*, and *Alternaria* sp.) as well as race 2 elicit a resistant response. Therefore, a general and inducible response may operate that ultimately serves as a defense mechanism (22,37).

An effect on the fungus prior to penetration, viz., a decrease in the number of appressoria as well as penetration, suggests that inhibitory compounds are released into the infection court on the surface of the leaf. In several host-pathogen systems, diffusates from infected leaves have been shown to inhibit conidial germination of the pathogen (2,16,17,21,32). Therefore, it is tenable to suggest that fungitoxic compounds, released when host tissue is contacted by a nonpathogen or a potential pathogen, influence the outcome of the host-pathogen interaction. We have obtained evidence that an inhibitory compound is released from susceptible leaves inoculated with conidia of *H. carbonum* race 2 or other fungi. This inhibitor reversibly prevents germination and germ tube elongation of race 1 conidia in vitro and in vivo and prevents infection and lesion formation in the host when incubated simultaneously with conidia of race 1. The involvement of this inhibitory compound is discussed in greater detail by Cantone and Dunkle (4)

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