Isolation of Phytoalexins from Corn with Monogenic Resistance to Helminthosporium turcicum

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ABSTRACT

Two phytoalexins, A1 and A2, were found in monogenic resistant corn. These phytoalexins were produced when spore suspensions of *Helminthosporium turcicum* from corn were incubated on detached corn leaves. An isolate of *H. turcicum* from Johnson grass did not stimulate production of these phytoalexins. Similar phytoalexins were also isolated from extracts from infected, intact leaves. No phytoalexin was found in diffusates or extracts from infected, susceptible corn or noninfected corn leaves of either genotype. Phytoalexin A1 has a paper chromatography R_F value of 0.87 in upper phase of BAW, and an ultraviolet absorption spectrum with a maximum at 280 nm. Phytoalexin A2 has an R_F value of 0.97 and an ultraviolet absorption spectrum with a maximum at 270 nm. Both phytoalexins are blue fluorescent compounds. Reaction with diazotized sulfanilic acid reagent indicates that they are probably phenolic. Fungal isolates varied in their response to phytoalexin. At the same phytoalexin concn, spores of a highly pathogenic isolate germinated better than those of a weakly pathogenic one. Also, highly pathogenic isolates induced the formation of a higher concn of phytoalexins than did weakly pathogenic isolates. Results indicate that monogenic resistance of corn to pathogenic isolates of *H. turcicum* probably is chemical in nature and is due to phytoalexins produced in response to infection by this fungus. Phytopathology 60:1071-1075.

Additional key words: *Zea mays*, leaf drop-diffusate technique, leaf extracts, bioassay of phytoalexin, dosage response effects.

Monogenic resistance to *Helminthosporium turcicum* Pass. in corn is characterized by the formation of chlorotic lesions, delayed necrosis of host tissue, and inhibited fungus sporulation. This type of resistance is expressed by young seedlings as well as by older plants. The resistance is expressed in the field or greenhouse following natural or artificial inoculation (3, 5). To date, no known naturally occurring American isolates of *H. turcicum* are capable of producing susceptible wilt-type lesions on monogenic resistant corn (3). In addition, more than 100 isolates of *H. turcicum* collected throughout the world were avirulent when tested on monogenic resistant seedlings (6).

Initial symptoms appearing as yellowish, light-green flecks are similar on both resistant and susceptible leaves. Reaction of resistant and susceptible corn differs in 3-7 days when these flecks begin to enlarge. On resistant leaves, the flecks elongate parallel to the veins and remain narrow; most lesions develop slowly and remain yellow to brown. In contrast, flecks on susceptible leaves enlarge rapidly and develop into large, wilt-type lesions (3).

A pathological histology study (4) revealed that initial stages of infection by *H. turcicum* are similar in both resistant and susceptible plants. Fungal hyphae penetrate into the xylem 2-3 days after inoculation, and flecks begin to appear on leaves at this time. The first histological differences between resistant and susceptible corn appear when the fungus reaches the xylem. In susceptible leaves, lesions enlarge by the growth of hyphae in the xylem and from the xylem into healthy sheath and chlorenchyma. Rapid cell death follows, resulting in large, wilt-type lesions. Wilting, however, fails to develop in lesions of the monogenic resistant leaves. Fungus mycelium is restricted to xylem vessels or tracheids, and hyphae grow poorly in the mesophyll tissue. Consequently, lesion enlargement is minimized. Morphological features that could explain the difference in hyphal growth in resistant and susceptible corn were not observed.

Our previous studies showed this chlorotic lesion type of resistance to be related to the production of phytoalexin (7). Diffusates and leaf extracts from infected monogenic resistant corn contain substances inhibitory to spore germination. No inhibitory substances were detected in diffusates or leaf extracts from infected susceptible corn or from noninoculated resistant or susceptible corn.

It is well-known that phytoalexins are produced as a result of the interaction of two metabolic systems, host and parasite, and that the phytoalexin inhibits the growth of pathogens (2). In many instances, a plant appears to be resistant only if it produces phytoalexin in a concn sufficient to inhibit the growth of the parasite (8).

Recent progress in biochemistry permits new approaches to the study of the biochemical nature of plant resistance. Our studies showed that the host-pathogen system of monogenic resistance to *H. turcicum* in corn is an excellent model to provide a better understanding of the chemical nature of plant resistance. This paper reports further progress in studies of phytoalexin production in corn with monogenic resistance to *H. turcicum*.

MATERIALS AND METHODS.—*Host and pathogen.*—The plant materials used in this study were the dent corn single crosses ROh43Ht × RB37Ht and Oh43 × B37. Resistant inbreds ROh43Ht and RB37Ht were derived from the susceptible (ht) inbreds Oh43 and B37, respectively, by introducing the dominant gene *Ht* for chlorotic lesion resistance to *H. turcicum* into them by repeated backcrossing for five generations,

followed by selfing and selection. The resistant single cross is therefore genetically similar to the susceptible single cross except for having the gene *Ht*.

Isolates of *H. turcicum*, obtained from field-infected corn plants, were tested for their pathogenicity. Highly pathogenic isolates, i.e., those that produced numerous large lesions on corn, were cultured on potato-dextrose agar (PDA) and spores harvested from 10- to 12-day-old culture plates. Newly harvested spores and sporulating cultures were stored at 5 C and used within 30 days, since longer storage or repeated transfers on PDA reduced pathogenicity. To maintain high pathogenicity, susceptible corn plants were inoculated with the stored cultures, and isolations made from these infected plants. Weakly pathogenic isolates from corn were also used to a limited extent. An isolate of *H. turcicum* from Johnson grass (*Sorghum halepense* [L.] Pers.), which is nonpathogenic to corn, was also included in parts of the study.

Preparation of inoculum.—For inoculation of detached leaves, spore suspensions were prepared from culture plates. A 9-cm diam sporulating culture was cut into small blocks and placed in a flask containing 70 ml of distilled water. The flask was then shaken gently, and the spore suspension filtered through cheesecloth. Fresh spore suspensions of the above concentration were prepared for each experiment. For inoculation of seedlings, the sporulating PDA culture of each plate was added to 70 ml of distilled water and blended for 2 min in a Waring Blendor. The spore suspension was then filtered through a 100-mesh sieve.

Isolation of phytoalexin.—Two different methods of obtaining corn phytoalexin were used. In the first, the leaf drop-diffusate technique was used (7). Sections of leaves from greenhouse seedlings were placed on 15 ml of a solution containing 5% sucrose and 20 ppm kinetin in 15-cm diam plates. Several drops of a spore suspension were placed on each leaf section with a small pipette. After specified time periods under a light intensity of 150-200 ft-c at room temp, the drops were collected and spores and germ tubes removed by centrifugation. These solutions were then dialyzed against distilled water at 5 C for 12 hr. The dialyzed solutions were evaporated to dryness in a flash evaporator. The residues were dissolved in 70% methanol and stored at 3-5 C for further purification. To obtain a large quantity of diffusate, 50 plates were prepared for each set of experiments. In each 15-cm diam plate, 10 ml of spore suspension in drops was placed on the closely spaced sections of resistant leaves. Approximately 400 ml of diffusate was collected from 50 plates 3 or 4 days after inoculation.

In the second method, leaf extracts were obtained from infected plants grown in the greenhouse. Resistant corn plants at the fifth to sixth leaf stage were sprayed with inoculum and incubated for 16 hr at 100% relative humidity. Inoculated leaves were harvested 10-12 days after inoculation, when symptoms were clearly expressed. Approximately 2 kg of infected leaves were harvested from each set of experiments. The leaves were extracted for 12 hr by placing them in 10 liters of boiling water which slowly cooled to room temp. The extracts obtained were filtered and concd to 500 ml. An equal volume of acetone was added to the extract, and the resulting precipitate washed three times with an equal volume of acetone. Acetone solutions were combined and concd to dryness. The residue was suspended in distilled water and dialyzed against distilled water. Dialyzed solutions were concd to dryness and resuspended in 70% methanol. This solution was then stored at 3-5 C.

For column chromatography, 250 g of Sephadex G-25 were slowly added to warm distilled water in a large beaker with continuous stirring. The beaker containing the Sephadex gel was then placed in a 60-C water bath and allowed to stand for 3 hr. The supernatant and fines were discarded, and a 5% methanol solution was added to the swollen Sephadex gel at five times its volume. The Sephadex gel was stirred and allowed to settle, and the fines and supernatant were again removed. This procedure was repeated three times, and the gel packed in a 5- × 70-cm column.

Paper chromatography was done on Whatman 3 MM paper with the upper phase of *n*-butanol, acetic acid, and water (4:1:5) (v/v) (referred to as BAW) as the ascending or descending solvent. Absorption spectra in the ultraviolet and infrared regions were determined for the phytoalexins.

Bioassay of phytoalexin.—The diffusates or leaf extracts were bioassayed by a spore germination test. Drops of spore suspensions were placed on plexiglass slides. After the spores had settled, the water was withdrawn with a small pipette and replaced by a solution to be tested for fungitoxicity. After overnight incubation in a moist chamber at room temp, the spores were killed and stained with an iodine potassium iodide solution (2 mg I_2 + 20 mg KI/ml water), and the percentage of germination was determined. As a control, drops of distilled water were used in most experiments, since diffusates or extracts from infected susceptible leaves or noninfected corn showed no effect on spore germination in all previous experiments.

RESULTS.—*Time sequence of phytoalexin production.*—To determine the time sequence of phytoalexin production, spore suspensions of both pathogenic (corn) and nonpathogenic (Johnson grass) isolates of *H. turcicum* were placed on sections of resistant and susceptible leaves, and the diffusate was withdrawn at daily intervals for 4 days. This experiment was repeated several times with little variation among results. Data obtained from one of these experiments is given in Table 1. The diffusates from detached susceptible leaves inoculated with both pathogenic and nonpathogenic isolates did not inhibit spore germination. Also, the diffusates from detached resistant leaves inoculated with the nonpathogenic isolate did not inhibit spore germination. Only diffusates collected 3 or more days after detached resistant leaves were inoculated with the pathogenic isolate significantly inhibited spore germination. When different corn isolates were used in these experiments, maximum reduction in spore germination occurred in the diffusate collected 4 or more

TABLE 1. Percentage germination of a pathogenic (corn) and a nonpathogenic (Johnson grass) isolate of *Helminthosporium turcicum* spores in diffusates obtained at daily intervals from detached leaves of resistant and susceptible corn inoculated with these isolates

Incubation day	Spore germination in diffusates			
	Corn isolate		Johnson grass isolate	
	Resistant	Susceptible	Resistant[c]	Susceptible[c]
	%	%	%	%
1	93.3[a]	93.7	83.5	85.1
2	91.3	91.4	85.5	83.9
3	24.4**[b]	92.7	83.9	86.9
4	13.8**	90.9	82.7	82.2
Control	91.0[d]		83.5[d]	

[a] Each value is the mean of the three replications.
[b] Different from control at the 1% level of significance.
[c] Resistant or susceptible to corn isolate; only chlorotic flecks form on either genotype when inoculated with the Johnson grass isolate.
[d] Spore germination in distilled water.

days after inoculation. It also appeared that highly pathogenic isolates induced a greater total amount of phytoalexin than did weak isolates.

Isolation of phytoalexin in diffusates.—A 0.5-ml quantity of concd diffusate was spotted in a narrow band across a 25- × 25-cm sheet of Whatman 3 MM chromatography paper. After development of 24 sheets in BAW, the chromatograms were dried and the area between the origin and the solvent front was cut into five equal strips. Substances on each strip were eluted with 70% acetone. Eluted substances were dried and the residues were resuspended in distilled water. These individual fractions were then bioassayed. Only the two fractions with R_F values between 0.6 and 1.0 caused a significant inhibition of spore germination when compared with water controls. These two fractions, upon rechromatography in BAW and electrophoresis in 0.01 M potassium phosphate buffer at pH 7.0, yielded only two fluorescent compounds which were inhibitory to spore germination. All other repurified fractions proved to be noninhibitory. These two inhibitory fractions were designated A1 and A2. A1 has an R_F value of 0.87 and an ultraviolet absorption maximum at 280 nm; A2 has an R_F value of 0.97 and an ultraviolet absorption maximum at 270 nm (Fig. 1).

Isolation of phytoalexin from extracts of greenhouse inoculated plants.—Dialyzed solutions were concd and adjusted to pH 4.0 with 5% HCl. These solutions were washed four times with ether. The ether extracts were dried and resuspended in 5% methanol and used for column chromatography. A concd 30-ml sample equivalent to 2 kg of infected leaf tissue was placed in the column, and 5% methanol used as a developing solution. The flow rate was 100 ml/30 min. Twenty fractions, 100 ml each, were collected in 125-ml flasks. These fractions were dried, resuspended in 50 ml of distilled water, and then bioassayed. Fractions 8, 9, and 10 all caused a significant reduction in spore germination. Maximum reduction of spore germination occurred in fraction No. 9. Untested portions of fractions were pooled and evaporated to dryness. The residue was dissolved in 70% methanol for paper chromatography. The solution was spotted in narrow bands across a 46 × 57 cm sheet of Whatman 3 MM chromatographic paper. After development of 10 such sheets by descending chromatography in BAW, the chromatograms were dried and the area between the origin and the solvent front was cut into five equal strips running across the 6 cm width of the sheets. The fractions with R_F values between 0.60 and 1.00 caused a significant reduction in spore germination. The eluates were pooled and chromatographed again in the same manner as that followed in the purification of diffusates. Two inhibitory substances were obtained. Charac-

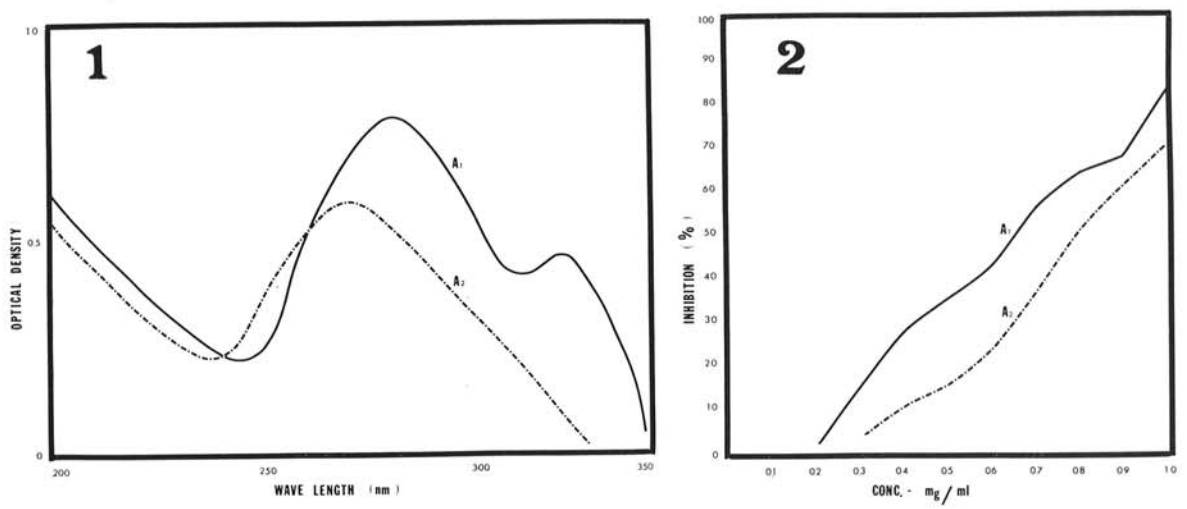

Fig. 1-2. **1)** Ultraviolet absorption spectra of phytoalexins, A1 and A2, produced in resistant corn leaves infected with *Helminthosporium turcicum*. R_F values: A1 = 0.87; A2 = 0.97. **2)** Effect of phytoalexins, A1 and A2, on inhibition of *H. turcicum* spore germination.

teristics of these substances were identical to those which had been obtained and designated from the diffusates. A1 fluoresces blue and A2 fluoresces light yellowish-blue under ultraviolet light. Reaction with diazotized sulfanic acid reagent indicates that the inhibitory compounds probably are phenolic. Eluates containing A1 and A2 compounds were evaporated to dryness and residues were resuspended in a small volume of ethanol. These solutions were air-dried and placed in a vacuum oven at 25 C. The A1 compound formed a brown powder and A2, a yellowish-brown powder. These dried powders were soluble in water and methanol. Yields of relatively pure compounds were approximately 40 mg/kg of infected leaves for both A1 and A2.

Dosage response effects.—Inhibitory effects of the isolated A1 and A2 compounds on spore germination were tested, and dosage response curves for each were plotted (Fig. 2). Significant inhibitions of spore germination were obtained at concn of 300 μg/ml and above. It should be noted that development of the fungus is hindered but not stopped in the resistant host. When compared at the same dosage, A1 showed a stronger inhibition of spore germination than A2. Various corn isolates of *H. turcicum* were used in these experiments. When compared with weakly pathogenic isolates, more spores of a highly pathogenic isolate germinated at a given concentration of phytoalexin. Preliminary observations indicate that spore germination of the nonpathogenic Johnson grass isolate and *H. maydis* was inhibited. Germination of *Puccinia sorghi* urediospores, however, was little affected by the phytoalexin.

Discussion.—Monogenic resistance in corn to *H. turcicum* is associated with the production of phytoalexin(s) when resistant leaves are infected with pathogenic isolates of the fungus. Susceptibility is attributed to insufficient production of phytoalexin or to the lack of it. The nearly isogenic host stocks used in the study indicate that phytoalexin production is related to the presence of gene *Ht* in the plant. Noninfected plants do not contain inhibitory substances. There is, in resistant leaves, a positive correlation between the time of detectable phytoalexin production and observable inhibition of fungus growth. Both of these occur 3-4 days after infection. Apparently identical inhibitory compounds A1 and A2 were obtained from both diffusates and extracts from infected resistant leaves. Although present evidence indicates that the gene *Ht* functions in a similar manner when introduced into various genetic backgrounds, the time, rate, and/or amount of phytoalexin production may differ. These effects, along with phytoalexin production in other corn lines expressing chlorotic-lesion resistance to *H. turcicum,* remain to be studied. Some of these lines contain different genes for resistance, while others contain dominant genes indistinguishable from the gene *Ht* used in this study.

Diffusates collected from resistant and susceptible corn plants inoculated with a nonpathogenic isolate of *H. turcicum* from *Sorghum halepense* did not inhibit spore germination. Spores of this isolate germinate well on corn leaves, yet they do not produce lesions or phytoalexin on these plants. Only minute chlorotic flecks appear on infected leaves. This suggests that genes for pathogenicity to corn are absent in this isolate, and that continued growth of the fungus in the resistant leaf is prerequisite to phytoalexin production. It has been suggested that it may be possible to obtain biotypes of *H. turcicum* that are virulent on corn by hybridizing such a nonpathogenic isolate with an isolate pathogenic to corn (6). At present, however, there are no known naturally occurring races or isolates of *H. turcicum* that carry the necessary complement for virulence to monogenic resistant corn. Apparently, the *Ht* gene conditions resistance against a broad spectrum of *H. turcicum* isolates.

Isolates of *H. turcicum* from corn fields have shown some degree of variation in their ability to induce the formation of the corn phytoalexins and in their sensitivity to them. All appear to induce identical phytoalexins in the monogenic resistant plants tested. However, highly pathogenic isolates induce a higher concentration of phytoalexins than do the weak ones. Also, the spore germination percentage of a highly pathogenic isolate is higher than that of a weak isolate when tested at the same phytoalexin concn. This suggests that the basic response of monogenic resistant corn to *H. turcicum* is to produce phytoalexins, but the rate of phytoalexin production and the quantity produced may vary with the fungal isolate.

The phytoalexin chemical structure is apparently determined by the genotype of the plant, not by the genotype of the parasite. The parasite genotype can determine only a certain degree of sensitivity to a given phytoalexin (1). In many cases, with other hosts and pathogens, more than one phytoalexin has been isolated from infected plant tissue (8). Compounds A1 and A2 may not differ greatly in their chemical characteristics, although A1 inhibits spore germination more than A2 at equal concn. The infrared spectrum indicates the two compounds are very similar, and reaction with diazotized sulfanilic acid reagent indicates that both of them may be phenolic. Further study on phytoalexins A1 and A2 and phytoalexins that may be obtained from other resistant corn may help to determine whether gene *Ht* induces the formation of more than one phytoalexin or that a gene or genes closely linked to *Ht* are activated to produce a specific phytoalexin in this host-pathogen interaction.

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