Disease Determinant of Helminthosporium maydis Race T

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ABSTRACT

Extracts of corn leaves having *cms*-T cytoplasm infected with *Helminthosporium maydis* race T exhibited a differential toxicity to resistant (normal) and susceptible (*cms*-T cytoplasm) corn similar to that of the pathotoxin (HmT-toxin) produced by the fungus in vitro and similar to the reaction of the two corn cytoplasm types to the fungus itself. The extracts obtained from the resistant corn leaves infected with race T, with the exception of a very susceptible inbred, did not show the selective toxicity. The toxic activity of extracts from corn inbreds with large lesions was greater than that from inbreds with small lesions. The physical and chemical characteristics of the toxic fraction extracted from infected leaves are similar to the pathotoxin produced by *H. maydis* race T in culture.

This study indicates that the toxic fraction of the infected leaf extract contains pathotoxin, which is the disease-determinant of southern corn leaf blight.

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Additional key words: normal cytoplasm, lesion size, seedling root assay, toxic fraction of infected leaf extract.

Several pathogen-produced toxins have been identified as factors in plant disease development. Such toxins are referred to as pathogen-produced determinants of disease in order to avoid confusion between pathogen-produced substances and host-produced substances involved in disease resistance (7).

The host-selective pathogenicity of *Helminthosporium maydis* Nisikado & Miyake race T to corn (*Zea mays* L.) containing *cms*-T (Texas male-sterile) cytoplasm is now well known (1, 2, 8). Race T produces a host-specific pathotoxin in vitro which is highly toxic only to susceptible corn having *cms*-T cytoplasm (1). This pathotoxin is capable of inducing lesions on detached leaves of susceptible plants. Our studies (3) suggest that the inheritance of the selective pathogenicity and the specific pathotoxin production of race T to *cms*-T corn plants are monogenic, whereas the degree of pathogenicity expressed and the amount of pathotoxin produced are polygenic in inheritance.

This study was carried out to determine whether the pathotoxin produced by race T plays a major role in the disease development of southern corn leaf blight.

MATERIALS AND METHODS.—*Host stocks.*—Five pairs of susceptible and resistant dent corn inbreds, nearly isogenic except for *cms*-T cytoplasm in the susceptible corn, were grown in the greenhouse at 24-27 C. The inbreds were Oh07^T, WF9^T, Hy2^T, B37^T, and Mo17^T and their normal cytoplasm counterparts Oh07, WF9, Hy2, B37, and Mo17, respectively. These inbreds and the single crosses P-A-G SX29^T and SX29 with and without *cms*-T cytoplasm were used in the seedling root assay test for presence of pathotoxin.

Preparation of inoculum and inoculation of plants.—Susceptible seedling leaves heavily infected with race T in the greenhouse were collected 10 to 12 days after inoculation. A portion of the collected leaves was cut into small sections (5-7 cm) and placed in moist covered plates (15 X 150 mm). They were then incubated at 24-27 C for 48-72 hr in the dark. The remaining infected leaves were air-dried at 24 C and kept at 3-5 C for subsequent inoculations. Leaves with sporulating lesions were transferred from the moist plates to a flask containing distilled water. The flask was then shaken and the spore suspension filtered through cheesecloth. Spore suspensions, adjusted to 30,000-50,000/ml in distilled water, were sprayed onto resistant and susceptible corn in the fifth- to sixth-leaf stage grown in 12- by 24-inch rectangular flats and incubated in a saturated atmosphere for 16 hr in the dark.

Measurement of lesion size.—Lesions on infected leaves were measured longitudinally when lesions were clearly expressed, 10 to 12 days after inoculation. Three lesions selected at random on the fourth leaf from each of four infected corn plants were measured for each inbred. The data were expressed as the mean length of the 12 lesions.

Seedling root assay.—The tolerance of 10 corn inbreds to the pathotoxin was estimated by a corn seedling root assay. Seeds from each of 10 inbreds were soaked in distilled water for 8-12 hr at 24-27 C. The seeds were then surface disinfested with 0.1% $HgCl_2$, washed 3 times with sterile distilled water, and placed embryo-side down on moist paper in 150-mm-diam plates. Seeds were germinated for 24-48 hr, and only those with a uniform root length of 5 mm were used for the assay. Ten germinated seeds were placed embryo side down in petri dishes containing 20 ml of pathotoxin filtrate obtained from *H. maydis* race T grown in culture (Lim & Hooker, *unpublished data*). The increase in length of primary root development was measured for each seedling after 48 hr of darkness at 24-27 C. The data were expressed as the mean elongation of the 10 roots.

Preparation of leaf extracts and characterization of the toxic fraction.—Fifty g of infected leaves were harvested from each of the 10 corn inbreds 12 days after inoculation. The leaves of each inbred were extracted separately in 500 ml of boiling water for 10 min and left at 24-27 C for 16 hr. The extract was filtered through cheesecloth and concentrated in a flash evaporator to 50 ml. An equal volume of absolute methanol was added to the concentrated extract, and the resulting precipitate was washed 3 times with absolute methanol. Methanol was removed from the supernatant liquid by evaporation, and the liquid was concentrated to dryness. The residue was suspended in distilled water and dialyzed against distilled water for 72 hr at 3 C. The dialysate was collected every 24 hr and combined. Each dialysate was concentrated to 50 ml, and the weight:volume ratio was kept constant for all extracts. Dilution series were made with distilled water and assayed for the pathotoxicity against resistant (SX29) and susceptible (SX29^T) seedlings. The assay end point (the dilution that gave 50% inhibition of root elongation of *cms*-T corn as compared to roots of normal corn in the leaf extracts) was determined for each extract.

Untested portions of dialysate from the extracts of infected susceptible leaves were combined and concentrated to one-tenth the original volume, which was equivalent to 250 g of infected leaves. The concentrated dialysate was adjusted to pH 2.0 with 0.6 N HCl and extracted 4 times with an equal volume of butanol. The butanol phase was concentrated to dryness and dissolved in 5% methanol at 20 times concentration of the original volume. Column chromatography was carried out using Sephadex G-15 packed with 5% methanol in a 2.5 X 45 cm column. Two ml of concentrated butanol phase were layered on the column and eluted with 5% methanol at a flow rate of 10 ml/40 min. Thirty fractions were collected following the void volume. These fractions were dried, resuspended in 40 ml (approximately the original volume) of distilled water, then assayed for host-specific toxicity against resistant and susceptible seedlings. Untested portions of toxic fractions were combined and concentrated to dryness. This residue was dissolved in 75% methanol for further separation using paper chromatography. Ascending and descending paper chromatography was

TABLE 1. Lesion sizes on corn seedling leaves infected with *Helminthosporium maydis* race T 10-12 days after inoculation

Inbred line	Longitudinal lesion size (mm)			
	cms-T cytoplasm corn		Normal cytoplasm corn	
	Mean[a]	Range	Mean	Range
Mo17	3.0 a[b]	2.5-4	1.9 f	1.5-2.5
B37	6.8 b	5-9	2.3 fg	1.5-3.0
Hy2	7.6 c	6-9	2.5 ag	1.5-3.5
WF9	8.3 d	6-11	2.5 ag	1.5-3.5
Oh07	12.0 e	9-14	3.1 ah	2.0-4.0

[a] Each value is the mean of 12 lesions–three lesions on the fourth leaf from each of four infected seedlings.

[b] Means not followed by the same letter are significantly different at the .05 level of probability as determined by Duncan's multiple range test.

TABLE 2. Elongation of primary roots starting with an initial length of 0.5 mm when incubated for 48 hr in a pathotoxin solution from *Helminthosporium maydis* race T

Inbred line	Primary root elongation (mm)			
	cms-T cytoplasm corn		Normal cytoplasm corn	
	Pathotoxin	Check[a]	Pathotoxin	Check
Mo17	12.6 a[b,c]	39.7 c	34.8 d	41.2 c
B37	13.8 a	42.1 c	34.8 d	41.8 c
Hy2	12.6 a	41.0 c	36.4 d	40.0 c
WF9	11.8 a	42.5 c	35.6 d	42.0 c
Oh07	6.0 b	40.6 c	33.6 d	41.6 c

[a] Sterilized Fries culture solution.

[b] Each value is the mean of 10 seedling roots.

[c] Means not followed by the same letter are significantly different at the .05 level of probability as determined by Duncan's multiple range test.

done on Whatman 3 MM paper with the *n*-butanol, acetic acid, and water (4:1:5 v/v) (BAW). Portions of the toxic fraction from paper chromatography were exposed to ninhydrin and also used to determine the absorption spectra in the ultraviolet with a Beckman Model DB-G Spectrophotometer.

RESULTS.—*Pathogenicity expressed by the lesion size.*—When inoculated with *H. maydis* race T, all five corn inbreds containing *cms*-T cytoplasm produced significantly larger lesions than their normal cytoplasm counterparts (Table 1). Significant differences in lesion size were also found among the inbreds containing *cms*-T cytoplasm. Differences in lesion size among inbreds containing normal cytoplasm were less extensive. No significant difference in lesion size was found between inbreds Mo17^T and Oh07. These results indicate that Mo17^T is more resistant to race T than the other inbreds with *cms*-T cytoplasm used in this study, and that Oh07^T is the most susceptible. These relationships between the two inbreds also hold when they have normal cytoplasm.

Seedling root assay with pathotoxin.—In a pathotoxin filtrate obtained from the fungus growing in culture, root elongation of the inbreds containing *cms*-T cytoplasm was greatly inhibited when compared with that of their normal cytoplasm counterparts (Table 2). Root elongation of Oh07^T seedlings was inhibited to a greater degree than that of other inbreds. No significant differences in the inhibition of root growth were found among inbreds having normal cytoplasm. Root elongation of both *cms*-T and normal cytoplasm corn was significantly inhibited in pathotoxin when compared to roots in a check solution of Fries culture medium. No significant differences in root elongation in the check solution were found among all the inbreds having *cms*-T and normal cytoplasm. These results indicate that resistant and susceptible corn inbreds react in a similar way to the pathotoxin and to the pathogen. There was a negative correlation ($r = -0.78$) between lesion size of inbreds having *cms*-T cytoplasm and root elongation. The seedling root assay, however, failed to distinguish as many differences among inbreds with *cms*-T cytoplasm as did a determination of leaf lesion size.

Toxic specificity of the leaf extract.—All extracts from infected inbreds having *cms*-T cytoplasm exhibited pathotoxic specificity to cytoplasms similar to that of the pathotoxin produced by the fungus in culture. With the exception of the very susceptible inbred Oh07, extracts obtained from infected leaves having normal cytoplasm showed no specificity to the two cytoplasms.

Assay end points were determined for extracts that showed specificity (Table 3). The extract of Oh07 had an assay end point of 1:60. Various assay end points were found among pathotoxic extracts from inbreds having *cms*-T cytoplasm. The extract of highly susceptible inbred had the highest assay end point. The extract of the highly resistant inbred had the lowest assay end point. There was a highly significant correlation ($r = 0.95$, $P < .01$) between lesion size and assay end point of pathotoxic leaf extracts obtained from the infected susceptible corn. The assay end point was, however, negatively correlated ($r = -0.85$) with the primary root elongation in pathotoxin produced by the fungus in culture.

Physical and chemical characteristics of the toxin fraction.—The toxin fraction obtained from the extract of susceptible corn leaves infected with race T was dialyzable and thermostable. The host-specific activity was not altered after extraction in boiling water for 10 min. Sephadex fractions 18, 19, and 20, corresponding to 2.25-2.50 times void volume, all showed inhibition of root growth of the susceptible when compared to resistant corn. Maximum inhibition of root growth occurred in fraction No. 19. When toxin fractions were combined and chromatographed on paper, the fraction with R_F value of 0.95 inhibited the growth of susceptible seedling roots. This spot was eluted and had an ultraviolet absorption max at 270 nm and fluoresced light yellow under long wave ultraviolet irradiation. This spot also gave a slight reaction with ninhydrin at

TABLE 3. Assay end point of the toxic filtrate extracted from leaves of corn seedlings infected with race T at 10-12 days after inoculation

Inbred line	Assay end point			
	Leaf extract of *cms*-T cytoplasm corn		Leaf extract of normal cytoplasm corn	
	Infected	Noninfected	Infected	Noninfected
Mo17	1:25[a]	[b]		
B37	1:60			
Hy2	1:70			
WF9	1:12			
Oh07	1:17		1:60	

[a] Dilution series of leaf extracts that gave 50% inhibition of root elongation of susceptible ($SX29^T$) seedlings as compared to roots of resistant (SX29) seedlings. Distilled water was used to dilute the concentrated 50-ml leaf extract which was obtained from 50 g of leaves from each inbred.

[b] No differences in root elongation were found between susceptible and resistant seedlings in the leaf extracts where no data is given.

70 C for 15 min. The dried toxin fraction is soluble in methanol, butanol, and water, but is not soluble in chloroform, carbon tetrachloride, or carbon disulfide. These characteristics and the host specificity of the leaf extract to resistant and susceptible corn are similar to those of the pathotoxin produced by the fungus in culture (Lim & Hooker, *unpublished data*).

DISCUSSION.—The physical and chemical characteristics and the host-specific toxicity of the leaf extract obtained from susceptible corn infected with race T are similar to those of the host-specific pathotoxin produced by the fungus in culture (7, Lim & Hooker, *unpublished data*). Apparently, the extractable concentration of the toxic fraction in susceptible corn infected with race T is variable depending upon disease susceptibility as measured by lesion size, which in turn may reflect degree of fungus growth. A higher concentration of the toxic fraction was obtained from susceptible corn with large lesions than from resistant corn with small lesions. The pathotoxin produced by the fungus in susceptible corn retains its activity and appears to play a major role in the destruction of plant cells and in the production of the characteristic symptoms of the blight disease. It has been previously reported (4) that pathotoxin produced by race T interacts selectively with mitochondria extracted from susceptible corn (*cms*-T) cytoplasm.

The leaf extract of resistant corn containing normal cytoplasm did not exhibit the host-specific toxicity except when extracts were obtained from the susceptible inbred Oh07. The concentration of extractable pathotoxin in resistant corn infected with race T is very low, and unless the plant produces large lesions, it does not significantly affect plant growth.

To explain resistance to toxin in plants, Scheffer & Pringle (6) suggested, based on their data with *H. victoriae* toxin, that resistant plant tissue simply lacks a toxin receptor or toxin sensitive-point. However, there is no direct evidence yet to prove their interpretation. An earlier explanation was that toxin resistance is based on ability of resistant tissue to inactivate the toxin (5).

Race T pathotoxin interacts selectively with the cytoplasm and in particular with the mitochondria of susceptible cytoplasm (4). It appears to be the disease determinant, but its mode of action remains to be determined.

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