Differential Response of Sweet Corn Cultivars to Phytotoxic Water-Soluble Compounds from Culture Filtrates of *Exserohilum turcicum*

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ABSTRACT

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Phytotoxic substances were isolated from culture filtrates of *Exserohilum turcicum* grown in Fries' medium, or from susceptible corn plants infected with the fungus. The substances significantly inhibited chlorophyll biosynthesis in corn seedlings susceptible to *E. turcicum* but not in those resistant to the fungus. The phytotoxic substances were extracted from culture filtrates of eight different isolates of *E. turcicum*. A positive correlation was found between lesion size of isolates and phytotoxic activity. Lesion size and infection efficiency of *E. turcicum* on susceptible excised leaves pretreated with one or more phytotoxic substances increased by 119 and 30%, respectively, indicating that these substances may be a virulence factor.

Northern leaf blight of corn (*Zea mays* L.), incited by the fungus *Exserohilum turcicum* (Pass.) K. J. Leonard & E. G. Suggs, is one of the major diseases of corn in all the corn-growing areas of the world, including Israel (11,17,21). It causes necrotic elongated lesions in susceptible hybrids (3). Major dominant genes conferring qualitative resistance and polygenic quantitative resistance are used to control the disease (7,14,15). The *Ht1* gene conditions a chlorotic lesion response; the *Ht2* and *Ht3* genes condition a chlorotic lesion response with necrosis. The *HtN* gene prolongs the latent period (9). Races of *E. turcicum* that are virulent on corn possessing the *Ht* genes occur naturally in many growing areas (23). Races 1, 2, and 3 have the virulence formula (effective/ineffective host gene) *Ht1 Ht2 Ht3*/0, *Ht2 Ht3 HtN*/*Ht1*, and *Ht1*/*Ht2 Ht3*, respectively (16). Recently Leonard et al (10) proposed a new nomenclature for pathogenic races of *E. turcicum*, in which races 1, 2, and 3 become 0, 1, and 2.3, respectively. Yoka and Albertini (26) and Petitprez et al (18) reported that *E. turcicum* produces nonspecific toxins when grown in culture. Five other *Helminthosporium*-related species, all pathogenic to graminaceous hosts, are known to produce host-specific toxins (5). In other fungi, specific toxins were found to increase disease development but are not essential for pathogenesis (22). In this study, we present evidence that *E. turcicum* produces cultivar-specific toxic compounds associated with aggressiveness of the fungus toward susceptible corn hybrids.

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MATERIALS AND METHODS

Toxin extraction. Eight single-lesion isolates of *E. turcicum* collected from various locations in Israel were used in all the experiments. Race determination was done as previously described (1), using A619*Ht1*, A619*Ht2*, A619*Ht3*, and B37*HtN* as differential cultivars. Resistance and susceptibility were based on the presence or absence of chlorotic-type lesions. Only race 0 has been isolated in Israel (1). For isolation of toxin, 250-ml bottles containing 100 ml of modified Fries' medium (19) were inoculated with mycelial plugs of the fungus grown on lactose casein agar (24) for 14 days. Bottles were incubated at 25 C in growth chambers illuminated with cool-white fluorescent light at an intensity of 150 $\mu E\ m^{-1}\ s^{-1}$ for 12 hr/day. After 21 days of growth, the culture fluid was obtained by filtration through three layers of cheesecloth. The mycelia were dried at 60 C for 48 hr and then weighed. The culture filtrates were concentrated in vacuo at −50 C to 10–20% of their original volume with a lyophilizer (Virtis Company, Gardiner, NY). An equal volume of methanol was added, and the solution was stored overnight at 4 C. Precipitates were removed by filtration through Whatman No. 1 filter paper. Methanol was removed in vacuo at 45 C, and an equal volume of chloroform was added. The water phase was separated from the chloroform, and an equal volume of ether was added to the water phase. The ether was removed, and the water phase was used for bioassays. For the control treatments, the same procedure was done with modified uninoculated growth medium. This procedure was similar to the method used by Petitprez et al (18), with some modifications. For toxin isolation in vivo, sweet corn plants (cv. Jubilee) 21 days old were infected with an aggressive isolate of *E. turcicum*, as previously described (12). Five days after inoculation, 30-g samples of the infected as well as uninoculated leaves were chopped with a blender and treated with 100 ml of methanol. The material was extracted by heating at 50 C in a reflux condenser for 10 hr, then cooled and incubated overnight at 4 C. The methanol extracts were filtered through three layers of cheesecloth and through Whatman No. 1 filter paper. Water (100 ml) was added to the filtrate. Methanol was removed in vacuo at 45 C, and the water fraction was treated similarly to the in vitro extraction with chloroform and ether. This method was similar to that described by Vidhyasekaran et al (25), with some modifications.

Detached leaf bioassay. The bioassay was a modification of that used by Harel and Klein (6) and Rasmussen and Scheffer (20). Corn seedlings (Table 1) were grown in the dark for 10 days. The etiolated (first and second) leaves were excised above the coleoptile in the dark. The basal ends of excised leaves (1 g of fresh weight per duplicate sample) were placed in 5 ml of the experimental solutions in vials of 20 ml and held for 2 hr at 25 C in the dark. A small fan was used to increase transpiration and the uptake of the solutions. After the dark period, leaves were removed from the experimental solution and washed, and the basal ends were placed in water in vials. The leaves were then exposed to fluorescent light at 150 μE m^{-1} s^{-1} for 20 hr at 25 C to promote chlorophyll synthesis. After exposure to light, excised leaves were blotted dry and weighed. Chlorophyll was extracted and determined according to Jeffrey and Humphrey (8). Bioassays were performed with either the culture filtrate after the methanol extraction or the chloroform, ether, and water extractions.

In other experiments, corn seedlings were grown for 10 days in a growth chamber at 25 C, with a day length of 12 hr at a light intensity of 200 μE m^{-1} s^{-1}. Leaves were excised above the coleoptile. The basal ends of excised leaves were placed in the experimental solution in vials and held overnight at 25 C in the light. A small fan was used to increase transpiration. Leaves were placed on moist filter paper (adaxial surface upward) in plastic petri dishes (9 cm in diameter) and were inoculated with droplets (10 μl) of a conidial suspension (about 100 conidia per droplet), three droplets per leaf. After 7 days the number of successful infections and lesion sizes was recorded. For evaluation of lesion size and infection efficiency of isolates, sweet corn hybrids (Table 1) were grown in the greenhouse as previously described (13). Five-week-old plants with five or six leaves were inoculated with conidial suspensions prepared by washing conidia from 10-day-old cultures grown in an incubator at 25 C on lactose casein agar medium (24). Plants were inoculated by placing 10-μl droplets of the conidial suspension along the adaxial surface of the leaves (10 droplets per leaf, about 20 conidia per droplet). Inoculated plants were incubated overnight in a dew chamber at 20 C and then transferred to a growth chamber adjusted to 25 C. Infection efficiency (the number of lesions formed, as the percent of the number of sites inoculated) and lesion size were recorded 12 days after inoculation. Each experiment was conducted three times using at least 10 plants per isolate. The Waller-Duncan k-ratio t test was used for mean separation.

RESULTS

Area of lesions caused by individual isolates on corn plants (cv. Jubilee) ranged from 2.2 to 5.1 cm^2. Infection efficiency of isolates varied from 15 to 65% (Table 2).

In all hybrids containing *Ht1* genes chlorotic lesions were produced, whereas in the susceptible hybrids necrotic lesions were produced. The largest necrotic and chlorotic lesions were observed in hybrid 2570 and Super Sweet 8000, respectively (Table 3).

Inhibition of chlorophyll formation in etiolated leaves of the susceptible corn hybrid Jubilee treated with the water, chloroform, or ether extracts from culture filtrate of *E. turcicum* (isolate Biqat haYarden) was 50.0, 17.5, and −3.2%, respectively. No inhibition of chlorophyll formation was observed when leaves were treated with the ether extract (Table 1). A similar degree of inhibition of chlorophyll formation was observed in both susceptible and resistant hybrids when treated with the chloroform extract; the water extract inhibited chlorophyll formation significantly in susceptible hybrids but not in hybrids that contained *Ht* genes (Tables 1 and 3). The water extract from culture filtrates of three isolates of *E. turcicum* race 0 did not inhibit chlorophyll formation in etiolated leaves of hybrids containing *Ht1*, *Ht2*, *Ht3* or *HtN* genes, but it did inhibit chlorophyll formation of the near isogenic hybrids, which do not contain the *Ht* genes (Table 4). Similar trends were observed when etiolated leaves were treated with water extract from infected leaves, but at a lower intensity. Inhibition of chlorophyll formation in plants treated with water extract from infected leaves was 19% on the average (n = 10, P = 0.0206). Furthermore, in three of six resistant hybrids a significant increase in the amount of chlorophyll (Table 3) relative to the corresponding control treatment was observed. The response of the different hybrids was observed with water extract produced by each of the various isolates of the fungus (*data not shown*). The highest range of chlorophyll inhibition was observed in plants treated with water extract of isolate Biqat haYarden, and the lowest with water extract of Yad Mordekai (Table 2). A significant correlation was found between chlorophyll inhibition caused by

Table 1. Inhibition of chlorophyll production by extracts of culture filtrate of *Exserohilum turcicum* race 0 on a resistant (Jubilee) and a susceptible (Moore) sweet corn hybrid

Hybrid	Resistant gene	Extract	Inhibition of chlorophyll formation (%)[y]
Jubilee	None	Water	50.0 a[z]
Moore	*Ht1*	Water	−15.1 c
Jubilee	None	Chloroform	17.5 b
Moore	*Ht1*	Chloroform	19.2 b
Jubilee	None	Ether	−3.2 bc

[y] Inhibition of chlorophyll production in etiolated seedlings by extracts as percent of chlorophyll production of corresponding control treatment.
[z] Means followed by the same letter are not significantly different according to the k-ratio t test (P = 0.05; k = 100; n = 5). The experiment was repeated three times, with at least five replications for each experiment. Extracts were produced from culture filtrate of *Exserohilum turcicum* isolate Biqat haYarden, race 0.

Table 2. Inhibition of chlorophyll production by water extract of culture filtrates, lesion size, and infection efficiency of different isolates of *Exserohilum turcicum* on Jubilee sweet corn

Isolate[w]	Inhibition of chlorophyll production[x] (%)	Lesion size[y] (cm^2)	Infection efficiency (%)
Biqat haYarden	65 a[z]	5.1 a	65 a
Newe Ya'ar	62 ab	4.7 a	...
Bet She'an	60 ab	3.6 b	60 a
Bar-Ilan	55 ab	4.8 a	35 b
Givat Hayyim	48 ab	2.2 c	15 c
Ayyelet haShahar	45 b	3.0 bc	35 b
Revivim	25 c	2.4 c	...
Yad Mordekai	22 c	2.3 c	15c

[w] For location and fitness of isolates, see Leonard et al (10).
[x] Induced by water extract from 1 g of mycelium of each isolate; in etiolated seedlings, by water extract as percent of chlorophyll production of corresponding control treatment.
[y] Lesion size and infection efficiency were measured 10 days after inoculation of corn plants at the five-leaf stage.
[z] Means followed by the same letter within a column are not significantly different according to the k-ratio t test (P = 0.05, k = 100, n = 15 for lesion size and 5 for inhibition of chlorophyll formation and infection efficiency). Each replicate for infection efficiency evaluation consisted of 30 plants.

water extract of different isolates and lesion size, but not between chlorophyll inhibition and infection efficiency. Correlation coefficients and significance values (in parentheses) were 0.82248 (0.0122) for chlorophyll inhibition and lesion area, 0.28552 (0.5832) for chlorophyll inhibition and infection efficiency, and 0.1756 (0.7393) for lesion area and infection efficiency.

Lesion size of *E. turcicum* on susceptible excised leaves pretreated with the water extract from culture filtrate of *E. turcicum* isolate Biqat haYarden 24 hr before infection increased by 119%, as compared to the lesion size of similar leaves pretreated with the corresponding control extract. Similar treatments on resistant leaves did not significantly affect the lesion size (Table 5).

DISCUSSION

The variability of lesion size and infection efficiency caused by isolates of *E. turcicum* from various locations is a well-known phenomenon that was discussed in a previous paper (13). Toxic substances produced by *E. turcicum* induced inhibition of chlorophyll synthesis of etiolated leaves after exposure to light. This relatively rapid effect allows completion of an assay within 24 hr. The results of this study suggest that water-soluble toxic compounds produced by the fungus either in culture or in infected corn leaves may be cultivar-specific and a primary determinant of virulence or pathogenicity of the northern leaf blight syndrome. Inhibition of chlorophyll formation caused by the chloroform extract in both resistant and susceptible hybrids suggests the presence of non-specific toxins as found previously (18). Although the data presented do not provide enough evidence for the causal role of a toxin in northern leaf blight, the results do indicate that a significant relationship may exist between lesion size caused by isolates and toxin production, on the one hand, and susceptibility of sweet corn hybrids and sensitivity (in terms of inhibition of chlorophyll formation), on the other. This suggests that the toxic compounds may play an important role in the development of *E. turcicum* in sweet corn and in the resistance of sweet corn hybrids to the disease. Purification of the toxic compounds and more critical experiments of the role of toxins in disease development are in progress. The importance of toxins in the aggressiveness of the pathogen is strengthened by results showing that susceptible corn leaves treated with water extract prior to inoculation resulted in an increase in lesion size and number of lesions compared with untreated leaves. Higher doses of water extract were required to achieve similar results with leaves of resistant hybrids. Essentially similar results were obtained in a study of colonization of corn leaves by *Helminthosporium carbonum* Ullstrup (4) and of rice leaves by *H. oryzae* Breda de Haan (25). Validation of the role of cultivar-specific toxins as a disease determinant is ultimately strengthened by detection of toxic compound production in susceptible corn leaves. The high and significant correlation between inhibition of chlorophyll caused by water extract of different isolates and lesion size suggests that the toxin affects colonization of the pathogen but does not affect the infection process.

The distinct reaction obtained from resistant and susceptible plants suggests that water extract could be used to screen for resistance. The results obtained with the 12 sweet corn cultivars show that highly susceptible plants could be clearly separated from highly or moderately resistant plants. Modification of the technique or more observations might allow for better differentiation of hybrids of intermediate resistance. The increase in the amount of chlorophyll in resistant plants after exposure to light compared with the corresponding control is hard to explain. It may be attributed to the presence of cytokinins in the water extract (2). Further studies are needed to clarify this point. As race 0 is the only physiological race isolated in Israel, production of toxic substances by other physiological races was not examined.

Table 3. Inhibition of chlorophyll production by water extract of culture filtrate and lesion size and infection efficiency of *Exserohilum turcicum*[v] on various sweet corn hybrids

Hybrid	Genetic background[w]	Inhibition of chlorophyll formation (%)[x]	Lesion size (cm^2)[y]	Infection efficiency (%)[y]
Jubilee	S	55 a[z]**	5.5 a	65 a
Melody	S	34 ab**	2.5 b	55 a
FMX	S	27 b**	3.8 ab	65 a
Sugar Loaf	S	24 b**	2.2 b	20 b
2570	S	22 b	6.2 a	30 b
Super Sweet 7900	R	1 bc	*2.2 b	30 b
Super Sweet 7210	R	−9 c	*0.16 c	20 b
Super Sweet 7610	R	−15 cd	*0.1 c	20 b
Super Sweet 7620	R	−21 cd**	*0.2 c	15 bc
More	R	−30 cd**	*0.56 bc	10 c
Super Sweet 8000	R	−39 d**	*3.6 ab	10 c
LSD ($P = 0.05$)	...	30	2	10

[v] Isolate from Biqat haYarden; both infection and water extract are from the same isolate.
[w] S = susceptible to *E. turcicum*; R = resistant to *E. turcicum* due to the presence of *Ht1* gene.
[x] Inhibition of chlorophyll production in etiolated seedlings by water extract, as percent of chlorophyll production of corresponding control treatment. Numbers with two asterisks are significantly different from the control ($P < 0.05$).
[y] Lesion size and infection efficiency were measured 10 days after inoculation of corn plants at the five-leaf stage. Single asterisk indicates chlorotic lesions type.
[z] Means followed by the same letter within column are not significantly different according to the *k*-ratio *t* test ($P = 0.05$, $k = 100$, $n = 15$ for lesion size and 5 for inhibition of chlorophyll formation and infection efficiency). Each repetition for infection efficiency evaluation consisted of 30 plants.

Table 4. Inhibition of chlorophyll production by water extracts[x] of culture filtrates of three isolates of *Exserohilum turcicum* on sweet corn hybrids with and without *Ht* genes

Hybrid	Inhibition of chlorophyll formation (%)[y] Isolate[z] BH	BI	YM
A619	55 a	42 a	21 b
A619*Ht1*	−30 d	−22 d	−10 c
A619*Ht2*	−19 d	−14 cd	−5 c
A619*Ht3*	−15 d	−7 c	−7 c
B73	44 a	31 ab	20 b
B73*HtN*	−35 d	−25 d	−15 cd

[x] Or percent of chlorophyll production of corresponding control treatments.
[y] Means followed by the same letter are not significantly different according to the *k*-ratio *t* test ($P = 0.05$, $k = 100$). The experiment was repeated three times with at least five replications for each experiment.
[z] BH = Biqat haYarden, BI = Bar-Ilan, YM = Yad Mordekai.

Table 5. Lesion size of *Exserohilum turcicum*[x] on two sweet corn hybrids as affected by treatments with water extract from culture filtrates of different isolates of the pathogen[y]

Hybrid	Isolate	Lesion size[z]
Jubilee	Biqat haYarden	4.6 a
	Revivim	2.9 b
	Control	2.1 b
Super Sweet 7620	Biqat haYarden	0.35 ac
	Revivim	0.21 ac
	Control	0.15 ac

[x] Isolate Biqat haYarden.
[y] Corn seedlings were grown for 10 days with a day length of 12 hr. Leaves were treated with water extract 24 hr before infection. Lesion size was measured 7 days after infection.
[z] Means followed by the same letter (within hybrid) are not significantly different according to the *k*-ratio *t* test ($P = 0.05$, $k = 100$, $n = 15$).

All the differential hybrids containing one of the *Ht* genes show a resistant response to both *E. turcicum* race 0 and to the toxic compounds produced. The chlorotic response of hybrids containing *Ht1* genes as observed in both natural or artificial inoculations may be due to the presence of nonspecific toxins (18).

Further studies with toxic substances produced by the other physiological races of the pathogen are needed to understand the possible relationships between the toxic substances, physiological races, and host genotype.

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