Host-Specific Toxin Production by *Helminthosporium oryzae*

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ABSTRACT

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Culture filtrates of *Helminthosporium oryzae* contained a toxin which elicited the characteristic brown spot symptoms on rice leaves. Procedures for isolation and purification of the toxin, based on solvent extractions, adsorption on charcoal, alumina adsorption chromatography, gel filtration, and thin-layer chromatography are described. The R_f value and UV and IR adsorption spectra of the purified toxin revealed that it is a new toxin different from the three previously reported phytotoxins produced by *H. oryzae*. Rice cultivars susceptible to the pathogen were more sensitive to the toxin than the resistant cultivar. Sensitivity of rice cultivars and other plant species to toxin preparations corresponded to their relative susceptibility to the pathogen. When the virulent isolate lost its ability to produce toxin during repeated subculturing, it became nonpathogenic. Toxin added to the infection drops of the nonpathogenic isolate allowed invasion and colonization of rice leaves by the fungus. When the susceptible cultivars were sprayed with ferric chloride (10^{-2} M), they became resistant to both the pathogen and its toxin. The toxin could be isolated from the infected tissues. This toxin satisfies the criteria for consideration as a host-specific toxin and as a primary determinant of the disease.

Helminthosporium oryzae Breda de Haan causes brown leaf spot, a serious rice disease. The fungus is known to produce a phytotoxin, ophiobolin (13), which also has been named cochliobolin (19) or ophiobolin A (33) or cochliobolin A (4,5). This toxin reduced germinability of rice seeds and produced abnormalities in the seedlings (18); inhibited root and coleoptile growth (19) and respiration (20); induced electrolyte leakage in rice, beet-root, and maize (6,33); and affected cell membrane permeability (7). It also produced symptoms on nonhosts (14). The same toxin has been isolated from *Helminthosporium maydis*, *H. turcicum*, *H. zizaniae*, *H. leersi*, and *H. panici-miliacei* (10).

Two more phytotoxins, ophiobolosin A and B, were isolated from *H. oryzae* (16). Ophiobolosin A may be the same toxin which was identified as cochliobolin B (4,10) or a precursor of ophiobolin (4,5). Both toxins inhibited root growth of rice seedlings (16).

No host-specific toxin production by *H. oryzae* has been reported. But it is known that several nonspecific toxin-producing pathogens also produce host-specific toxins. *H. maydis* race T produces host-specific T-toxin besides the nonspecific ophiobolin (33). *H. carbonum* produces a host-specific toxin besides nonspecific carbtoxinine (25). *Alternaria mali* produces host-specific AM-toxins besides a nonspecific toxin, tenuazonic acid (11). Hence, we attempted to assess the possibility of host-specific toxin production by *H. oryzae* and our results show that *H. oryzae* produces a host-specific toxin which may be a determinant of brown spot disease in rice.

MATERIALS AND METHODS

Fungal isolates and pathogenicity. *Helminthosporium oryzae* was isolated from rice leaves collected from different parts of the Philippines. Single-spore isolates were maintained in potato-dextrose agar medium. We selected isolate I 46, the most virulent isolate, for our studies. For pathogenicity tests, the cultures were multiplied in sterilized rice husk medium (50 g of rice husk soaked in 20 ml of water and sterilized). The fungus sporulated profusely in this medium within 3–5 days.

Five rice cultivars (IR8, IR36, CH45, CO20, and IR1748-2-3-2-2-3) were used as test plants. Seed of these cultivars was obtained from the International Rice Germplasm Center at International Rice Research Institute, Philippines. The spore suspensions (approximately 50,000 spores per milliliter) were sprayed on 21-day-old plants. The inoculated plants were incubated in Percival dew chambers at 25 C for 48 hr and then moved to greenhouse benches. At 72 hr after inoculation, the disease intensity was assessed by scoring 25 leaves at random according to the Standard Evaluation System for Rice (1).

Isolation of toxin. One-liter Roux bottles containing 200 ml of modified Fries' medium (23) were inoculated with 1 ml of spore suspension of the fungus grown in rice husk medium for 5 days. After 21 days of growth under stationary conditions at laboratory temperature (25 ± 2 C), the culture fluid was obtained by filtration through three layers of cheesecloth. The culture filtrates were concentrated in vacuo at 50 C to 10% of their original volume. An equal volume of methanol was added and the solution was stored overnight at 5 C. Precipitates were removed by filtration through Whatman No. 1 filter paper. Methanol was removed in vacuo at 40 C and the water fraction was partitioned with three volumes of chloroform. The chloroform was evaporated in vacuo. The oily substance formed was weighed and dissolved in small quantities of methanol and the volume was adjusted with water to 10% of the original volume of the culture filtrate. This preparation was highly active in toxin bioassays and was used in most of the studies.

Purification of toxin. The toxin preparation in water was treated with activated charcoal U.S.P. - F.C.C. (Baker Chemical Co., Philipsburg, NJ) at 3% w/v, cooled to 4 C, stirred for 12 hr, and filtered through Whatman No. 1 filter paper. The filtrate did not have any toxic activity and was discarded. The toxin was desorbed from charcoal by washing on the filter paper with 500 ml of 5% methanol in chloroform. The eluate was evaporated to dryness in vacuo, weighed, and dissolved in 1 ml of chloroform.

Alumina (80–200 mesh; Fisher Scientific) was raised to red heat in an open vessel for 6 hr with stirring and then cooled in a vacuum desiccator. A column (1.5 × 25.0 cm) of alumina was packed in chloroform. The toxin sample (1 ml) was placed on top of the prepared column and eluted with 600 ml of chloroform. The chloroform eluate was dried in vacuo, weighed, and dissolved in 1 ml of chloroform.

Sephadex LH 20-100 was allowed to swell (24 hr) in chloroform-methanol-water (1:3:1) solvent and packed into a column. The Sephadex bed (1.5 × 25.0 cm) was washed with 500 ml of the solvent

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system before use. The toxin sample was placed on top of the column and eluted with the same solvent system. Ten-milliliter fractions were collected, the solvent was evaporated and the residue was dissolved in a small volume of methanol and then diluted with nine volumes of water. Toxic activity was detected by using the detached leaf bioassay. The toxin-containing fraction was dried in vacuo and weighed. Effectiveness of each step in the purification procedure was tested by obtaining the dry weight in each step and assessing the minimum concentration required to induce typical brown spot symptoms in rice leaves (cultivar IR36) in detached leaf bioassay.

The toxin containing fraction was further purified by thin-layer chromatography (TLC). The toxin was streaked on precoated TLC sheet (silica gel 60 F - 254, 0.2-mm layer thickness and aluminum support, size 20 × 20 cm; E. Merck, Germany) and developed to a 14-cm front with chloroform-methanol (100:5). The toxin was identified by spraying with 0.5% vanillin in sulfuric acid:ethanol (4:1). A purple spot appeared (R_f 0.89). The corresponding unsprayed zone was removed from the plate and eluted with chloroform and the toxin was bioassayed. The process was repeated three times. Purity of the toxin was checked by spraying the TLC plates with antimony pentachloride (20% in chloroform), phosphomolybdic acid (10% in ethanol), 2,4-dinitrophenylhydrazine (0.4% in 2 N HCl), and vanillin (0.5% in sulfuric acid:ethanol [4:1]). The TLC plates were developed with different solvent systems such as benzene:methanol (95:5), acetone-water (9:1), butanol:acetic acid:water (4:1:1) and chloroform:methanol (100:5).

The UV absorption spectra of the purified toxin were recorded in chloroform, methanol, and ethanol with a Hitachi 200-20 spectrophotometer. The IR spectrum was recorded with a Perkin-Elmer IR spectrophotometer.

Isolation of toxin produced in vivo. Leaves of 21-day-old rice cultivar IR8 seedlings were inoculated with isolate 46 as previously described. After 72 hr, 300 g samples of the infected as well as uninoculated leaves were chopped and treated with 600 ml of methanol. The material was extracted by heating at 65 C in a reflux condenser for 8 hr, then cooled and incubated at 4 C overnight. The methanol extracts were filtered through three layers of cheesecloth and through Whatman No. 1 filter paper. To the filtrate, 100 ml of water was added. Methanol was removed in vacuo at 40 C and the water fraction was partitioned with three volumes of chloroform. The water fraction was discarded and the chloroform was evaporated to dryness in vacuo. The oily substance that remained was dissolved in small volumes of methanol and diluted with water. The presence of the toxin was assessed by the detached leaf bioassay.

Detached leaf bioassay. Leaves of 21-day-old plants were detached and cut into 4-cm pieces. The tip and basal portion of the leaf were discarded. Each leaf section was placed over a glass slide and their ends were fixed with gummed tape (otherwise the leaf curled). An injury was made with the tip of a ball-point pen. The slide was kept inside a sterile petri dish lined with wet blotting paper. The toxin preparation in water was diluted to different concentrations. Fifty microliters of the diluted toxin preparations was placed on 4-mm-diameter filter paper disks which were placed on the injured leaf section. The petri dishes were incubated under laboratory conditions (25 ± 2 C). Light conditions did not affect the sensitivity of the assay. After 72 hr, symptom development was assessed. The maximum dilution at which visible symptoms were noticed was considered as the dilution end point of activity of the toxin (12).

Electrolyte leakage. The youngest fully expanded leaves of 21-day-old rice plants were used for the bioassay. The leaves were cut into small pieces (1 cm long and 5 mm wide) and rinsed, and random samples (200 mg) were enclosed in washed cheesecloth and placed in scintillation vials. Three milliliters of the diluted toxin preparation was infiltrated into the leaf sections in vacuo for 30 min. The leaf sections were then rinsed with water, and 4 ml of glass-distilled water was placed in each vial and incubated on a reciprocal shaker (100 strokes per minute) at 25 C. Conductances of ambient solutions were measured at intervals with a YSI model 31 conductivity bridge. Conductance in μmhos of the control leaves infiltrated with water was subtracted from the conductance of the toxin-infiltrated leaves to determine the increase in electrolyte leakage induced by toxin (12).

All the experiments were repeated two or three times.

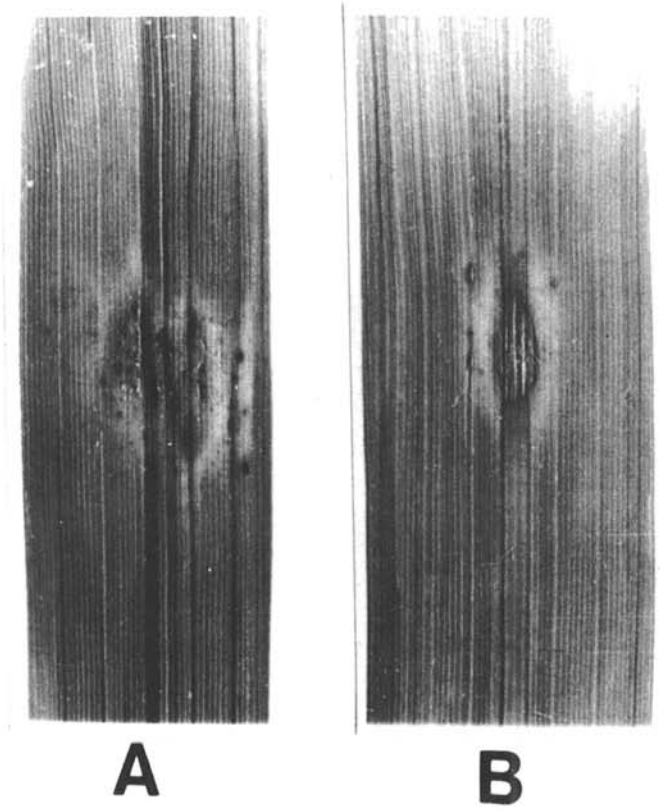

Fig. 1. Symptoms produced by **A,** *Helminthosporium oryzae* and **B,** its toxin on rice leaf.

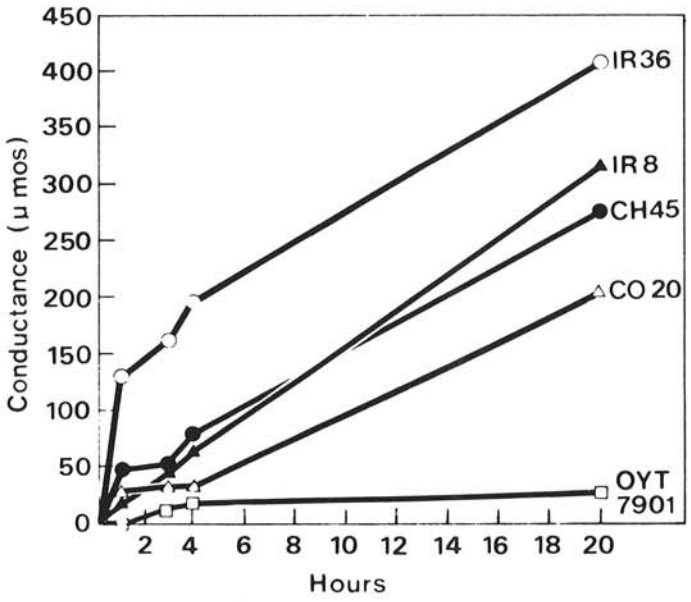

Fig. 2. Electrolyte leakage induced by toxin produced by *Helminthosporium oryzae* in susceptible (IR8, IR36, CH45, and CO20) and resistant (IR1748-2-3-2-2-3) rice cultivars. Least significant difference to compare any two means at $P = 0.05$ is 43.

RESULTS

Specificity of toxin. The toxin of the highly virulent isolate (I46) induced characteristic brown spots surrounded by yellow halo symptoms on the four susceptible (IR8, IR36, CH45, and CO20) and resistant (IR1748-2-3-2-2-3) rice cultivars. The symptoms were similar to those produced by the pathogen (Fig. 1). But when serial dilutions of the toxins were made, the differences between susceptible and resistant cultivars were discernible. The toxin induced symptoms on the susceptible cultivars even at 0.5–1.0 μg/ml, but no symptoms were observed in the resistant cultivar when the toxin was diluted to contain less than 100 μg/ml (Table 1).

The toxin was diluted to contain 50 μg/ml and the electrolyte leakage induced in the five cultivars was assessed. The toxin induced significantly more leakage in the four susceptible cultivars compared to the resistant cultivar, IR1748-2-3-2-2-3 (Fig. 2).

Host range of the fungus and activity of the toxin in those plant species was assessed by the detached leaf bioassay. Leaf sections of cultivar saba banana, Phil B026 sugarcane, UPL Sq5 sorghum, IPV var. 2 corn, Apollo tomato, Crocker tobacco, Dumaguete Long Purple eggplant, UPL Pn-2 peanut, and Pagasa 2 mungbean were inoculated with *H. oryzae* by preparing a spore suspension containing approximately 50,000 spores per milliliter and placing 50 μl of the suspension on 4-mm-diameter filter paper disks which were placed on leaf sections. The petri dishes containing the leaf sections were incubated as described previously under detached leaf bioassay.

H. oryzae could infect banana, sugarcane, sorghum, maize, and tomato but could not infect other hosts (Table 2). The toxin produced by the virulent isolate (I46) was diluted to contain 50 μg/ml and tested on leaves of these hosts by the detached leaf bioassay. The toxin induced typical brown spot symptoms on the plants which were susceptible to the fungus and did not produce any symptoms on the nonhosts of the fungus.

Purification of toxin. No toxin activity was detected in the precipitate after adding methanol or in the water fraction after partitioning with three volumes of chloroform. The toxin activity was detected in the chloroform fraction. All toxin was adsorbed by the charcoal and the water fraction was inactive. The 10% methanolic chloroform extract of charcoal contained eight different compounds, including the toxin fraction, which reacted to vanillin. In the alumina column, the toxin could be eluted with chloroform and at least 600 ml of chloroform was necessary to elute all of the toxin activity. This chloroform eluate contained three compounds reacting to vanillin. The toxin fraction appeared between the 40- and 70-ml fractions of the Sephadex LH 20-100 column. The void volume was 15 ml.

The toxin fraction migrated with a R_f of 0.89 (chloroform: methanol [100:5] solvent) on the TLC plates. The toxin reacted with vanillin (purple spot), phosphomolybdic acid (blue), 2,4-dinitrophenyl hydrazine (yellow), and antimony pentachloride (brown). When the unsprayed region was eluted and rechromatographed, no other spots were detected. In different solvent systems, the toxin migrated with different R_f values. In benzene-methanol it was 0.86, and in acetone-water and in butanol-acetic acid-water it did not leave the margin. The toxin eluted from TLC plates induced characteristic symptoms on rice leaves.

The effectiveness of each step in removing contaminating materials is presented in Table 3. The biological activity of the toxin increased with each step of purification.

The toxin was highly soluble in chloroform, methanol, ethanol, and acetone and sparingly soluble in water. The UV absorption spectrum of this compound is presented in Fig. 3. In chloroform, its maximum absorbance was at 242 nm, in methanol it was at 205 nm, and in ethanol it was at 207 nm. The IR spectrum in chloroform showed V_{max} 3,000, 2,950, 1,740, 1,309, 1,140, and 1,090 cm^{-1}.

Toxin as primary determinant of disease. When the highly virulent isolate was subcultured several times, its virulence decreased and subsequently it became nonpathogenic. The original isolate which was maintained in sterile soil at 4 C was compared with the less virulent one which produced only small specks and the nonpathogenic one which did not produce any symptoms. The nonpathogenic isolate did not produce the toxin while the less virulent isolate produced less toxin as indicated by the quantity of toxin collected by passing through Sephadex LH 20-100 columns. The activity of the toxin produced by the highly virulent and less virulent isolates was assessed by using the partially purified toxin, the volume of which was adjusted to 10% of the original volume of the culture filtrate as described previously. The activity was assessed both by detached leaf and electrolyte leakage bioassays. Both the bioassays also indicated that the less virulent isolate produced less toxin and the nonpathogenic isolate produced none (Table 4).

Spore suspensions (approximately 50,000 spores per milliliter) of pathogenic and nonpathogenic isolates were prepared and 50 μl of the suspension was placed on 4-mm-diameter filter paper disks which were placed on detached IR8 and IR36 leaves and incubated as described for the detached leaf bioassay. Spore germination, penetration, and mycelial growth inside the leaf tissue were assessed by clearing the leaves according to the method of Shipton and Brown (29). Spores of both isolates germinated well on the leaf surfaces, formed appressoria, and penetrated the epidermal layers at 12 hr after inoculation. At 24 hr, many infection pegs were observed in both the cases. At 36 and 48 hr after inoculation,

TABLE 1. Relationship between disease intensity caused by *Helminthosporium oryzae* and activity of toxin produced by it on leaves of susceptible (IR8, IR36, CH45, CO20) and resistant (IR1748-2-3-2-2-3) rice cultivars

Rice cultivars	Mean disease intensity[a]	Dilution end point of activity of toxin[b] (μg/ml)
IR8	6.8	0.5
IR36	6.3	0.5
CH45	5.0	1.0
CO20	8.0	1.0
IR1748-2-3-2-2-3	1.0	100.0

[a] Disease intensity in category values ranging from 0 to 9 according to the Standard Evaluation System for Rice (1).
[b] Minimum concentration of toxin needed to produce typical brown spot symptoms. Data are mean of three independent experiments.

TABLE 2. Host range of *Helminthosporium oryzae* and symptoms produced by its toxin on the hosts

Host	Disease intensity[a]	
	Fungus	Toxin
Rice	+++	+++
Banana	+++	+++
Sugarcane	+++	+++
Sorghum	+++	+++
Corn	++	++
Tomato	++	++
Eggplant	–	–
Peanut	–	–
Tobacco	–	–
Mungbean	–	–
Cowpea	–	–

[a] Symbols: +++ = very severe, ++ = moderate, and – = no symptoms.

TABLE 3. Effectiveness of each step in the purification procedure in isolation of *Helminthosporium oryzae* toxin

Step in purification	Dry weight[a] (mg)	Minimum concentration required to induce brown spot symptoms (μg/ml)
Chloroform	52	1.04
Charcoal	38	0.63
Alumina	9	0.11
Sephadex LH 20-100	5	0.045

[a] Dry weight of the toxic chemical obtained per liter of the culture filtrate.

extensive inter- and intracellular mycelial growth was observed only in the mesophyll tissue of leaves that had been inoculated with pathogenic isolate. The nonpathogenic isolate did not grow beyond the infection peg. The pathogenic isolate induced typical brown spot symptoms with yellow halo at 48 hr while the nonpathogenic isolate did not produce any visible symptoms. Spore suspensions of the nonpathogenic isolate (50,000 spores per milliliter) were prepared in diluted toxin preparation (50 μg/ml) and 50 μl of it was placed on the detached leaves as described previously. Spores of the nonpathogenic isolate germinated well on the leaves and produced appressoria within 12 hr. Numerous infection pegs were observed at 24 hr and extensive inter- and intracellular mycelial growth was observed at 36 hr. The growth rate of the mycelium of the nonpathogenic isolate was similar to that of the pathogenic isolate. It did not make any superficial saprophytic growth. Typical brown spot symptoms appeared at 48 hr. Similar brown spot symptoms appeared on leaves exposed to the toxin, but no mycelial growth was observed inside the leaf tissues (Table 5).

Several chemicals were screened to induce resistance against the disease. Ferric chloride at 10^{-2} M sprayed 48 hr before inoculation induced resistance against *H. oryzae* (Table 6). This resistance persisted up to 72 hr after treatment. Ferric chloride at lower dilutions (10^{-3} M or 10^{-4} M) was ineffective. Ferric chloride at 10^{-2} M did not inhibit spore germination or germ tube growth on the leaves. The leaves were detached from plants that had been sprayed with ferric chloride, and the activity of toxin produced by I 46 was assessed by detached leaf and electrolyte leakage bioassays. The "induced" resistant leaves showed reduced sensitivity to the toxin (Table 6).

The toxin isolated from diseased leaves induced characteristic brown spot surrounded by yellow halo symptoms on rice leaves. The toxin migrated with the R_f value of 0.89 when spotted on TLC plates and developed with chloroform:methanol (100:5). It showed the same UV absorption maximum absorbance of 242 nm in chloroform, 205 nm in methanol, and 207 nm in ethanol.

DISCUSSION

Phytotoxins that exhibit the same host specificity as the pathogen that produces them are classified as host-specific toxins (24). Unlike some plant pathogens, *H. oryzae* is not limited to a single host. The fungus infects 23 genera of grasses, including maize, barley, wheat, oats, Italian millet, and finger millet (21). The toxin we isolated also produced symptoms on many rice cultivars and other crops. But when the toxin dilution end point was considered, host-specificity of the toxin was discernible. In the case of many other toxins, resistant hosts were sensitive only to higher concentrations of toxins while susceptible hosts were sensitive to even lower concentrations (9,12,22,27,31).

The present toxin appears to be different from ophiobolin. The reported R_f value of ophiobolin in the chloroform:methanol system is 0.5 (33) while that of the present toxin is 0.89. The UV absorbance maximum of ophiobolin in chloroform is at 245 nm (17), in ethanol it is at 237.5 nm (13) or 238 nm (15), and in methanol it is at 236.0 nm (3). Our toxin shows absorbance maximum at 242 nm in chloroform, at 207 nm in ethanol, and at 205 nm in methanol. Our toxin is different from cochliobolin B which shows UV absorbance maximum at 237 nm in ethanol (4) and 239 nm in methanol (16), and R_f value of 0.49 in chloroform-methanol (11). The R_f value of ophiobolosin B was 0.70 in the chloroform-methanol and UV absorbance maximum was at 296 nm in ethanol (16). The IR spectrum of our toxin is quite different from that of ophiobolin A (3,15), cochliobolin B (4,16), or ophiobolosin B (16). Similar to our studies, cultural conditions and different isolation procedures had yielded host-specific and nonspecific toxins in *H. maydis* race T (33) and *H. carbonum* (25).

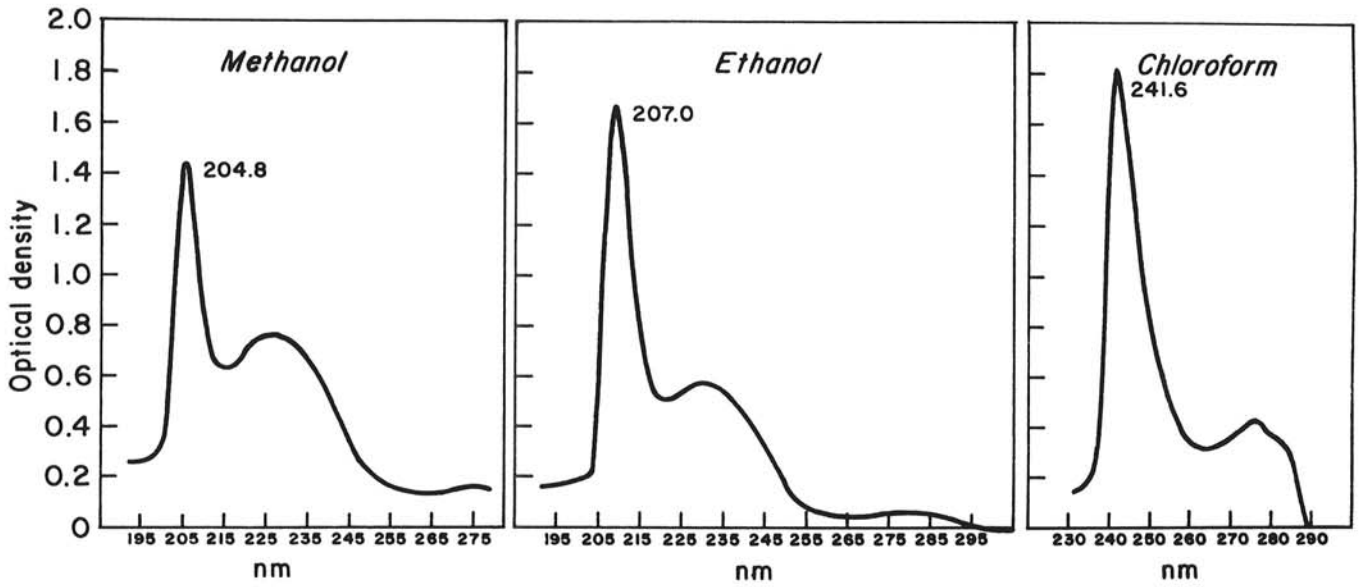

Fig. 3. Ultraviolet absorbance spectra of the toxin produced by *Helminthosporium oryzae* in methanol, ethanol, and chloroform.

TABLE 4. Relationship between virulence and toxin production by *Helminthosporium oryzae* isolates

Isolate	Disease intensity[a]		Dilution end point of activity of toxin		Increase in electrolyte leakage over water control (μmhos)[b]		Amount of toxin produced (mg per liter of culture medium)
	IR8	IR36	IR8	IR36	IR8	IR36	
Highly virulent	7.0	7.6	>500	>500	352 ± 15	430 ± 23	5.20
Less virulent	1.1	1.9	10	5	37 ± 5	60 ± 3	0.11
Nonpathogenic	0	0	–	–	0	0	0

[a] Disease intensity in category values ranging from 0 to 9 (1). Data are mean of two independent experiments.
[b] Differences were determined after 72 hr of exposure to toxin.

TABLE 5. Effect of toxin from *Helminthosporium oryzae* on development of nonpathogenic *H. oryzae* within rice leaf tissues

Treatment	Brown spot development[a]		Extent of fungal growth inside leaf tissue[b]	
	IR8	IR36	IR8	IR36
Virulent isolate	+	+	++	++
Nonpathogenic isolate	−	−	+	+
Toxin alone	+	+	−	−
Toxin + nonpathogenic isolate	+	+	++	++

[a]Symbols: −, No visual symptoms; and +, typical brown spots with yellow halo.
[b]Symbols: −, No mycelial growth; +, spore germination and infection peg only; and ++, abundant inter- and intracellular mycelial development within leaf tissues.

TABLE 6. Effect of ferric chloride spray on the disease intensity and activity of toxin produced by *Helminthosporium oryzae* in leaves of two rice cultivars

Treatment	Mean disease intensity[a]		Dilution end point of acivity of toxin[b] (μg/ml)		Increase in electrolyte leakage over water control (μmhos)[c]	
	IR8	IR36	IR8	IR36	IR8	IR36
Ferric chloride	1.6	1.0	20	10	10 ± 2	0
Water	7.3	7.5	>1	>1	215 ± 10	85 ± 16

[a]Disease intensity in category values ranging from 0 to 9 (1).
[b]Minimum concentration of toxin needed to produce typical brown spot symptoms. Data are mean of three independent experiments.
[c]Differences were determined after 72 hr of exposure to toxin.

To consider a toxin as a primary determinant of the disease, the loss of ability to produce toxin should lead to loss of pathogenicity (26). The present toxin satisfies this requirement. The less-virulent isolate produced less toxin and when the virulent isolate lost its pathogenicity, it also lost the ability to produce toxin. When the toxin was added along with the nonpathogenic isolate, the fungus developed within the host tissue as rapidly as the pathogenic isolate. In a similar study (31), it was observed that when a small amount of HV-toxin was added, the nonpathogenic mutant of *H. victoriae*, which did not produce toxin, developed throughout susceptible leaves just as rapidly as did the pathogenic *H. victoriae*. Essentially the same results were obtained in a study of colonization of corn leaves by *H. carbonum* (8). Topical application of toxin was necessary for successful infection of *Populus tremuloides* by spores or mycelium of *Hypoxylon mammatum* (28).

Many chemicals are known to induce resistance against *H. oryzae* in rice and ferric chloride is one of them (32). Our studies provide additional evidence that the toxin is a primary determinant of the disease since the plants became insensitive to the toxin when they became resistant to the fungus. Similar to our studies, application of nitrate-nitrogen increased resistance to symptom development in tomato infected with *Fusarium oxysporum* f. sp. *lycopersici* and membranes of cells from plants grown at high doses of nitrate nitrogen were less sensitive to the toxin, fusaric acid (2).

Validation of the role of host-specific toxins as primary disease determinants ultimately is strengthened by detection of toxin production by the pathogen in susceptible host plants (30) and we isolated the toxin from infected rice leaves. These studies suggest that the toxin from *H. oryzae* may be host-specific and a primary determinant of the brown spot disease.

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