

# Disease-Induced Toxin Production in *Helminthosporium oryzae*

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## ABSTRACT

The pathogenicity of a healthy and two diseased isolates of *Helminthosporium oryzae* was compared on Nato rice seedlings. Healthy fungus grew vigorously on sterilized soil, and caused typical *Helminthosporium* seedling blight. The diseased isolates grew poorly on soil, but caused pronounced inhibition of seedling growth and more severe disease than that caused by typical *H. oryzae*. Roots of rice seedlings were only slightly inhibited in undiluted culture filtrates of healthy *H. oryzae* and uninhibited at dilution 1:5. Roots were inhibited completely at dilution 1:10 of culture filtrates of the diseased isolates, and

inhibited 80% at dilution 1:100. Fifty per cent root inhibition occurred between dilutions 1:100 and 1:1,000 of toxin concentrate solutions. Dilutions made in vials after removal of the toxin concentrates caused root inhibition greater than 50% at  $10^{-8}$ . Culture filtrates of diseased *H. oryzae* sprayed on flowering rice panicles caused marked discoloration and a mean sterility of 32%, whereas culture filtrates of healthy *H. oryzae* caused almost no discoloration of panicles and a mean sterility of 17%. *Phytopathology* 61:420-424.

Two diseased cultures of *Helminthosporium oryzae* Breda de Haan (*Cochliobolus miyabeanus* Ito & Kuri-bay) were obtained from a group of isolations from infected rice seedlings. The causal agents of the diseases were transmitted to healthy *H. oryzae*; the transmissions were similar to those described for the diseases of *H. victoriae* (3) and *Agaricus bisporus* (1) and the vegetative death character of *Aspergillus glaucus* (2).

It became of interest to compare the pathogenicity of a diseased and healthy fungus system in which toxin production was not involved in the disease syndrome. Preliminary tests indicated no toxin activity in media that supported growth of healthy or type *H. oryzae*; however, Orsenigo produced symptoms of *Helminthosporium* seedling blight in rice with culture filtrates and autolysates of *H. oryzae*. A substance considered to be the toxin was purified from culture filtrates of the fungus (7). Oku (6) purified a substance toxic to rice seedlings from leaves infected with *H. oryzae*, but no toxin activity was demonstrated in crude preparations. The results reported here demonstrate toxin production by the diseased isolates of *H. oryzae*, but not by healthy *H. oryzae*.

**MATERIALS AND METHODS.**—Healthy *H. oryzae* (H-1) and the diseased isolate designated D-1D were obtained from infected rice seedlings. Diseased isolate D-1L occurred as a variant sector in colonies of D-1D. Once the agents of diseased isolates D-1D and D-1L were transmitted to H-1 and subcultured, all were regarded as diseased and healthy cultures of isolate 1 of *H. oryzae*. Isolates D-1D and D-1L were routinely propagated from transfers of H-1 inoculated by hyphal contact with the diseased isolates. Healthy H-2 of *H. oryzae*, isolated from a leaf lesion, was identical in culture type to H-1.

All inocula for the comparative pathogenicity trials were grown on potato-dextrose broth (PDB). Five-day-old mycelial mats of H-1, D-1D, or D-1L were blended in 1:10, w/v, of water. Thirty-five Nato rice grains were placed in dishes that contained 15 g of sterilized soil infested with 5 ml of the diseased or healthy *H. oryzae* inoculum.

Toxin activity was determined by a method similar to the one used by Luke & Wheeler (5). Diseased and healthy *H. oryzae* were grown in 20 ml of PDB in 250-ml flasks. Culture filtrates were prepared by pouring the broth of 5-day-old cultures through two layers of cheesecloth. Serial dilutions of the culture filtrates were made directly into dishes. Rice grains were pregerminated on soil and washed individually in running tap water. Seven seedlings with coleoptiles approx 1 mm in length were placed in the various dilutions to be tested. Inhibition of root elongation was used to measure toxin activity.

The toxin was concentrated by treatment of culture filtrates of diseased *H. oryzae* with anhydrous ether, 3:1, v/v. Where the toxin concentrates were assayed, 300 ml of culture filtrate were treated with 100 ml of ether. The ether phase was separated, the ether allowed to volatilize, and the toxin redissolved in 25 ml of distilled water. This preparation is hereafter referred to as the "toxin concentrate". Serial dilutions of the toxin concentrates were assayed similar to those of the culture filtrates. Dilutions were also made in vials that had contained the toxin concentrates. The toxin concentrate was first emptied from a vial, and an entire dilutions series made in that single vial. One ml of the toxin concentrate was returned to the vial with 9 ml of distilled water. This mixture was poured into a dish for assay and represented dilution  $10^{-1}$ . One ml of dilution  $10^{-1}$  was returned to the same vial with 9 ml of distilled water, and this dilution represented  $10^{-2}$ . The method of dilution was continued through dilution  $10^{-8}$ .

**RESULTS.**—Transmission of the agents of two diseased isolates of *H. oryzae* was tested by hyphal contact inoculation of healthy or type *H. oryzae*. Hyphal contact between diseased and healthy colonies was almost entirely accomplished by growth of the healthy mycelium; diseased colonies grew slowly and were sharply defined. Few symptoms of disease developed in the inoculated, once healthy, *H. oryzae*; and therefore, transmission was not readily evident. Evidence for transmission was obtained (i) by isolation of infected mycelium from the inoculated *H. oryzae* colonies (such transfers were taken

at a distance from the diseased inoculum that permitted no chance of contamination); and (ii) by the extremely low transmission per cent detected when a different healthy isolate of *H. oryzae*, H-2, was inoculated with D-1L and D-1D.

Mycelial transfers of the inoculated, once healthy *H. oryzae* were taken at a distance of 1 to 2 cm from the diseased inoculum and placed into fresh plates of PDA. The transfers were taken from the advancing edge of the inoculated colonies, 7 to 10 days after hyphal contact with the diseased inoculum. Of 120 transfers from each of H-1 and H-2 inoculated with D-1L, 63 and 4 developed diseased colonies, respectively. An equal number of transfers were made from H-1 and H-2 inoculated with D-1D; 45 and 7 developed diseased colonies, respectively. Colonies of D-1D were dark gray, with smooth to finely irregular edges, whereas colonies of D-1L were light-gray-to-white, with hyphal strands that formed irregularly stippled edges (Fig. 1).

*Pathogenicity on rice.*—The pathogenicity of diseased and healthy *H. oryzae* was compared on Nato rice seedlings. The healthy fungus grew vigorously on soil and produced typical *Helminthosporium* seedling blight (Fig. 2-A). Infected seedlings often turned brown and were killed, but surviving plants had necrotic lesions on the shoots, and were stunted compared to the healthy control plants in noninfested soil.

A disease unlike typical *Helminthosporium* seedling blight was produced by the diseased isolates of *H. oryzae*. The diseased isolates grew poorly on soil, and appeared not to attack the rice seedlings. In soil infested with diseased fungus, growth of rice seedling was, however, more strongly inhibited than was that of seedlings in soil infested with healthy *H. oryzae*. The shoots usually grew 2 to 3 mm, curved downward, and turned brown in color. The roots failed to grow except for an occasional root that grew above the soil surface. Such roots became necrotic at all points of contact with

infested soil containing the diseased fungus. These observations indicated that the diseased fungus might be producing a toxin exclusively.

*Bioassay of the toxin.*—Preliminary tests indicated that culture filtrates of the diseased isolates of *H. oryzae* produced symptoms in rice seedlings identical and equally severe to those produced by heavy concentrations of diseased fungus inoculum. Also, roots were damaged more by the culture filtrates than the shoots. Therefore, a root bioassay was used to compare root elongation in culture filtrates of diseased *H. oryzae* with root elongation in culture filtrates of healthy *H. oryzae*.

In four experiments, roots of rice seedlings were only slightly inhibited in undiluted culture filtrates of healthy *H. oryzae* and uninhibited at dilution 1:5 and greater dilutions. Culture filtrates of the isolates of diseased fungus completely inhibited root growth at dilutions up to 1:10; at dilutions of 1:50 and 1:100, root elongation was inhibited 90-95% and 80%, respectively (Fig. 3, 4).

*Concentration of the toxin.*—Attempts were made to concentrate the toxin by extraction of culture filtrates of diseased *H. oryzae* with certain organic solvents. Preliminary results indicated that the toxin was most stable in anhydrous ether. Bioassays of the concentrated toxin preparations indicated only slight increases in toxin activity over those of culture filtrates. Fifty per cent root inhibition occurred between dilutions  $10^{-2}$  and  $10^{-3}$  of the concentrated toxin regardless of the starting volume of culture filtrate used for extraction with ether. However, when an entire dilution series was made in a single vial after removal of the concentrated toxin preparations, root inhibition greater than 50% occurred at dilutions much greater than  $10^{-3}$ . Toxin concentrates were prepared and the dilutions made as described above. In each of four experiments, root inhibition greater than 50% occurred at dilution  $10^{-8}$  (Fig. 5). Approximately the same root inhibition curve was obtained when a vial that had contained concentrated toxin

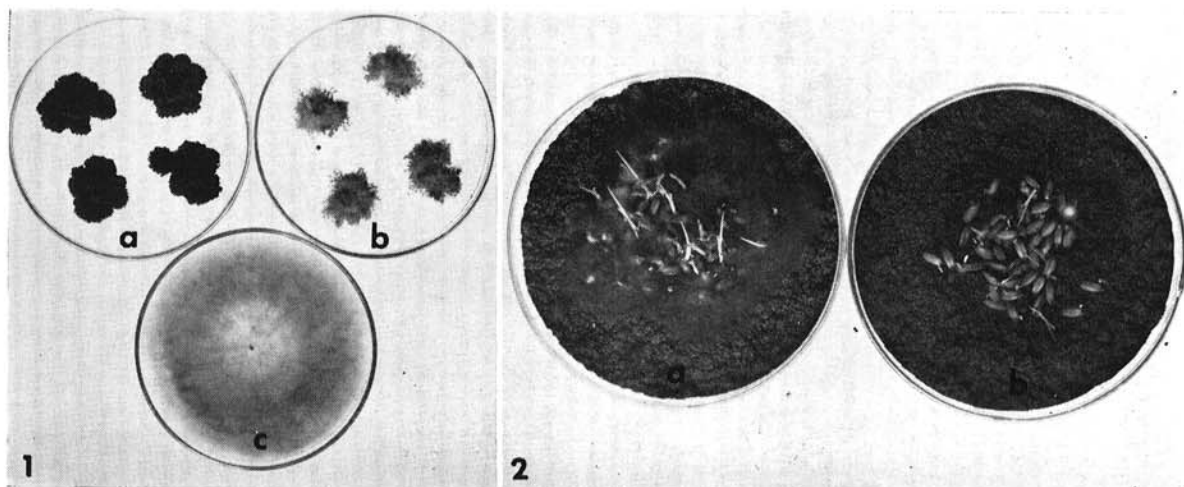


Fig. 1-2. 1) (a, b) Two diseased isolates of *Helminthosporium oryzae*, D-1D and D-1L, respectively. (c) Healthy *H. oryzae*. 2) (a) Rice seedlings in soil infested with healthy *H. oryzae* show typical *Helminthosporium* seedling blight. (b) Growth of diseased *H. oryzae* on soil was poor, but the seedling blight was severe.

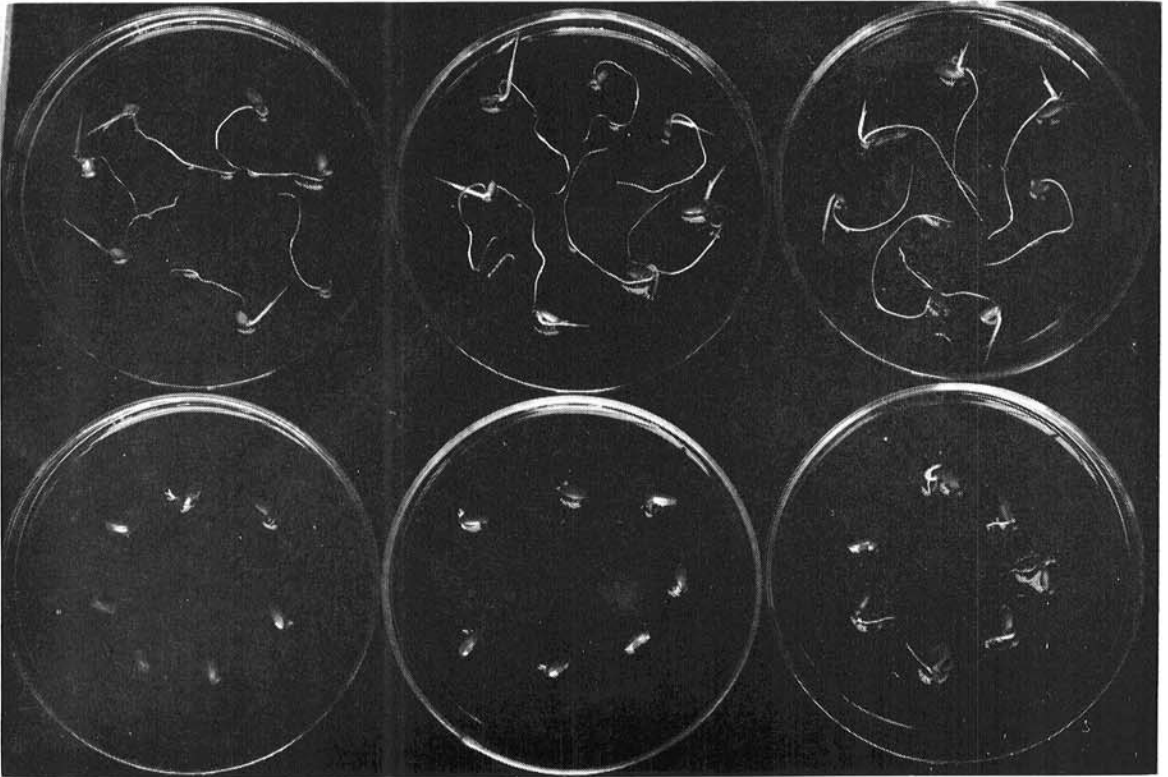


Fig. 3. Root bioassay of culture filtrates of healthy *Helminthosporium oryzae* (above) and diseased *H. oryzae* (below). From left to right, plates contain undiluted culture filtrates and dilutions 1:10 and 1:100.

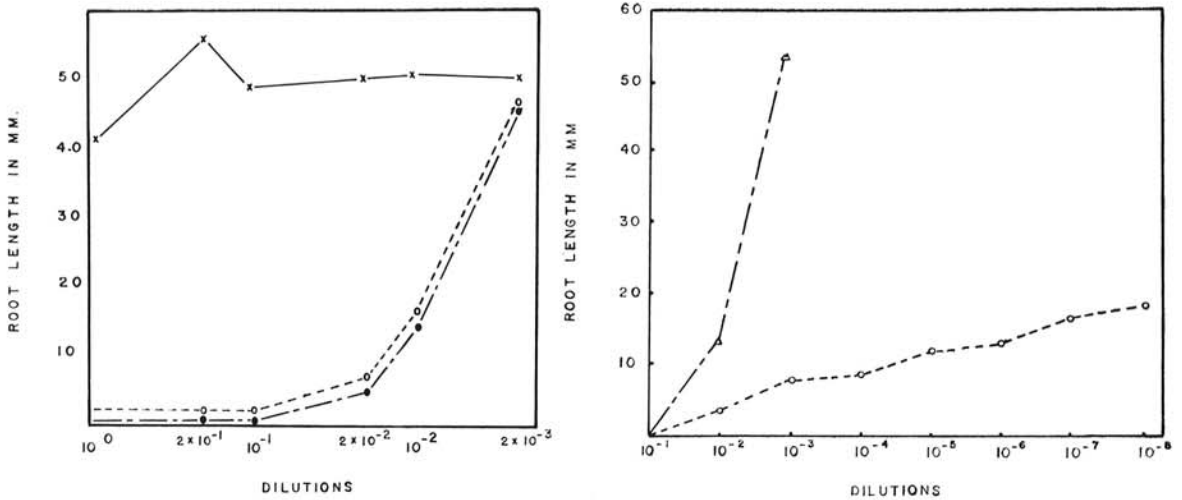


Fig. 4-5. 4) Root bioassay of dilution series of culture filtrates of healthy *Helminthosporium oryzae* (x); diseased isolate D-1D (●); and diseased isolate D-1L (○). Roots were uninhibited in culture filtrates of healthy *H. oryzae* but strongly inhibited in culture filtrates of the diseased isolates. 5) Roots were uninhibited at dilution 1:1,000 of the concentrated toxin (Δ). Dilutions made in a vial after removal of the toxin concentrate caused greater than 50% root inhibition at  $10^{-8}$  (○). Similar root inhibition curves were obtained when such a vial was rinsed 8 consecutive times with 9 ml of distilled water.

was rinsed 8 consecutive times with 9 ml of distilled water each time. The rinse corresponding to dilution  $10^{-8}$  caused root inhibition greater than 50%, as compared to the root length of the distilled water controls.

**Sterility and discoloration in rice.**—Much of the sterility and discoloration of rice panicles in Louisiana is considered to be caused by *H. oryzae*; *H. oryzae* was isolated readily from the brown lesions of spotted panicles, but not from badly discolored grains and sheaths. Flowering rice panicles were sprayed once with culture filtrates of diseased *H. oryzae* to determine whether the toxin enhanced sterility and/or discoloration. Panicles sprayed with culture filtrates of the diseased fungus had 83% of the grains discolored 50 to 100% and 33% sterility, whereas panicles sprayed with culture filtrates of healthy *H. oryzae* had 7% of the grains discolored 50 to 100% and 17% sterility (Fig. 6). Unsprayed control panicles had 3% of the grains discolored 50 to 100%, and 12% sterility (Table 1).

**DISCUSSION.**—Lindberg (4) reported 100% transmission of the agent of disease within a given isolate of *H. victoriae*, but relatively few transmissions from diseased of one isolate to healthy of a different isolate of *H. victoriae*. The agents of diseases D-1D and D-1L of *H. oryzae* were transmitted much more readily to H-1 than to H-2. It was concluded that the transfers from H-1 and H-2 inoculated with D-1D and D-1L were not contaminated with mycelium of the diseased fungus inoculum; otherwise the number of diseased transfers from the inoculated H-1 and H-2 would be about the same. The agents of the diseases in *H. oryzae* have not been identified.

Each generation or series of diseased cultures of *H. oryzae* was obtained by inoculation of healthy *H. oryzae*. Toxin production was never detected in culture filtrates of healthy *H. oryzae*, but toxin was produced by all diseased cultures. This is a clearly defined case of induction of toxin production; specifically, toxin production induced by diseased or abnormal physiology of the fungus.

The toxin in culture filtrates of diseased *H. oryzae*, or that extracted from such culture filtrates, produced similar symptoms in rice seedlings as that produced by the diseased fungus. The toxin was nonspecific, as it inhibited root growth of all cultivars of rice tested as well as rye (*Secale cereale* L.).

According to the root bioassays, much of the toxin extracted from culture filtrates of diseased *H. oryzae*

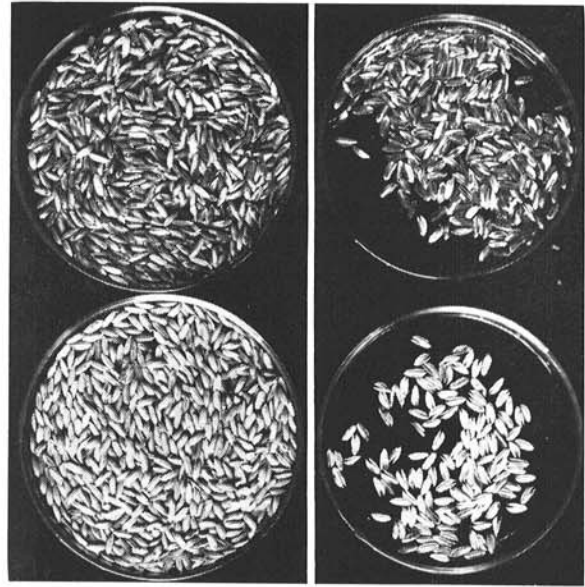


Fig. 6. Grains harvested from panicles sprayed with culture filtrates of diseased *Helminthosporium oryzae* (above); healthy *H. oryzae* (below). Plates at right contain empty hulls.

did not remain in solution but, apparently, adsorbed to the glass walls of the vial. The adsorbed toxin was eluted from the glass when dilutions were made in the vial after removal of the toxin concentrate.

Sterility and discoloration symptoms considered to be caused by *H. oryzae* but not reproducible with type *H. oryzae* were produced by culture filtrates of the diseased isolates of *H. oryzae*. Variant isolates of plant pathogens, especially diseased isolates, could prove to be the cause of many peculiar or exceptional plant disease symptoms encountered in nature.

Orsenigo demonstrated toxin activity in culture filtrates of *H. oryzae* and purified a toxin from such filtrates (7). We were unable to demonstrate any toxin activity in culture filtrates of healthy or type *H. oryzae*. It is possible that Orsenigo worked with a diseased isolate of *H. oryzae*, but his description of the growth of *H. oryzae* suggested that this was not the case. He obtained vigorous (profonditá) growth in liquid culture, whereas growth of our diseased isolates was poor compared to that of healthy *H. oryzae*.

TABLE 1. Discoloration and sterility of rice sprayed in the flowering stage with culture filtrates of healthy and diseased *Helminthosporium oryzae* as compared to unsprayed controls

Experiment	Panicles treated with culture filtrates of <i>H. oryzae</i>				Untreated panicles	
	Healthy fungus		Diseased fungus		% Grains discolored 50-100%	% Sterility
	% Grains discolored 50-100%	% Sterility	% Grains discolored 50-100%	% Sterility		
1	13	12	84	29	1	14
2	6	16	82	36	2	13
3	4	22	85	31	4	11
Means	7	17	83	33	3	12

## LITERATURE CITED

1. GANDY, DOREEN G. 1960. 'Waterstipe' of cultivated mushrooms. *Nature* 185:482-483.
2. JINKS, J. L. 1959. Lethal suppressive cytoplasm in aged clones of *Aspergillus glaucus*. *J. Gen. Microbiol.* 21: 397-409.
3. LINDBERG, G. D. 1959. A transmissible disease of *Helminthosporium victoriae*. *Phytopathology* 49:29-32.
4. LINDBERG, G. D. 1969. Separation of a mildly diseased from a severely diseased isolate of *Helminthosporium victoriae*. *Phytopathology* 59:1884-1888.
5. LUKE, H. H., & H. E. WHEELER. 1955. Toxin production in *Helminthosporium victoriae*. *Phytopathology* 45: 453-458.
6. OKU, H. 1964. Host-parasite relation in *Helminthosporium* leaf spot disease of rice plant from the viewpoint of biochemical nature of the pathogen. *In* Z. Király & G. Ubrizy [ed.] Host parasite relations in plant pathology. Hungarian Acad. Sci., Budapest Symp. p. 183-191.
7. ORSENIGO, M. 1957. Estrazione e purificazione della Cochliobolina una tossina prodotta de *Helminthosporium oryzae*. *Phytopathol. Z.* 29:189-196.