Studies of the Toxicity of *Helminthosporium maydis*¹

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Isolates of *Helminthosporium maydis* from blighted corn were tested for toxicity in mice, rats, swine, rabbits, microorganisms, and tissue culture. Extracts of grains, mycelia, and culture supernatant fluids killed mice on intraperitoneal (ip) injection, but were nontoxic on administration by mouth to swine. The toxin was partially purified and appears to be a glycerophospholipid. Histopathological examination revealed that the toxin acted as a severe irritant on ip injection, causing death in laboratory animals. In skin tests with rabbits, considerable exudation occurred, rather than necrosis.

*Helminthosporia* are ubiquitous fungi and many species are common plant parasites, particularly of the Gramineae. This latter family contains most of the world’s economically important grains—corn, wheat, sorghum, rice, oats, and barley. There are at least three *Helminthosporium* species that attack corn leaves: *H. maydis* Nishikado and Miyake, the causative agent of southern corn-leaf blight; *H. carbonum* Ullstrup, the causal agent of *Helminthosporium* leaf spot; and *H. turcicum* Passerini, the causal agent of northern corn-leaf blight. Besides corn, *H. turcicum* also has been reported capable of attacking sorghum (*Sorghum vulgare* Pers.) (9).

Southern corn-leaf blight, as caused by race O, has been known in the United States for a considerable period of time but was of minor economic importance. In 1969 and particularly in 1970, a new race (T) of this parasite appeared and reached epiphytic proportions in much of the corn-growing areas of the United States, causing severe economic losses. Corn carrying the factor for Texas male sterile cytoplasm (T) was vulnerable. It has been estimated that about 90% of the corn grown in the United States carried this factor in 1970. In addition to the leaves, race T also attacks the husks, penetrating the various layers to eventually grow on the kernels. Penetration also may occur in the tip and butt areas of the cob. At the tip in particular, the fungus rapidly proliferates to produce masses of black, multicelled conidia.

Corn infected with *H. maydis* may also be invaded by secondary fungi. In particular, we have isolated members of the genera *Fusarium*, *Trichoderma*, and *Alternaria*, including species which are known producers of mycotoxins (unpublished data).

Some of the corn plants that were blight damaged in 1970 were harvested early, ensiled, and fed to animals. Much of the corn, however, was permitted to mature and was harvested for commercial and “on farm” use. These uses of blighted corn pose potential problems, one of the more pertinent being: has mycotoxin synthesis occurred on blight-infected plants and kernels?

Hamilton et al. (4) found that *Cochliobolus carbonum* produces intracellular metabolites toxic to mice. This appears to be the only published study on the toxicity to mammals of secondary metabolites produced by corn-blight fungi, although it has been demonstrated that many individuals allergic to *Alternaria* and other fungal spores will show an allergenic response to *Helminthosporium* spores (3). There is only one report of *Helminthosporium* acting as a pathogen in man (2).

The present preliminary investigation was conducted to ascertain the capacity of several species of *Helminthosporium* to synthesize toxic substances. Mice, rats, swine, rabbits, and various bacteria, fungi, and tissue cultures were used as test organisms.

**MATERIALS AND METHODS**

**Microorganisms.** The *Helminthosporium* species used in this investigation were isolated from the
leaves, stems, and cobs of heavily blighted T-cytoplasm corn plants grown in Indiana and Illinois. In addition, cultures of *H. carbonum* races I and II and *H. maydis* races O and T were obtained from A. J. Ullstrup, Purdue University, Indiana. Cultures were maintained on Difco malt-yeast extract agar.

Toxicity of extracts of *H. maydis*-blighted corn plants. To test blighted corn plants naturally infected with *H. maydis* for the presence of toxins, 410 g of heavily infected leaves was extracted twice in a gallon-size Waring Blender with a total volume of 8 liters of chloroform-methanol (70:30); a similar extraction was made of noninfected corn leaves. Water was removed from the extracts with anhydrous sodium sulfate, and the solvent was evaporated in rotary vacuum evaporator at 40 C. Half of the residual solids from each extract were put into solution in 50 ml of olive oil. Two lots of five mice each (20-g Swiss Webster males) were injected intraperitoneally (ip) with 0.2 ml of the olive oil solutions twice a week for 3 weeks and then were maintained for a total of 6 months.

Toxicity of Helminthosporium isolates. A preliminary study was conducted to determine the toxicogenicity of a number of *Helminthosporium* isolates. Cultures were grown on 50-g quantities of corn and sorghum grain for 14 days at 28 C. The fermented grains were extracted with 500 ml of chloroform-methanol (70:30) by blending in a Waring Blender for 3 min. The solvent was recovered by filtration and the extracts were evaporated at 45 C in a flash evaporator; the residual solids in the extracts were dissolved in 2.0 ml of olive oil. Mice were injected (ip) with 0.1-ml portions. Based on the rapidity and number of mouse deaths, sorghum grain appeared to afford a better substrate for toxin production than corn. Among the cultures tested, *H. maydis* NRRL 3797 was arbitrarily chosen for further investigation.

Toxicity of extracts of *H. maydis* (NRRL 3797) grown on natural and artificial media. *H. maydis* NRRL 3797 was inoculated into 50-g quantities of whole corn and sorghum in 300-ml Erlenmeyer flasks and into 500 ml of YES medium (2% yeast extract, 15% sucrose) and Raulin-Thom (RT) medium in Fernbach flasks; cultures were incubated at 20 and 28 C for 18 days. Each flask of moldy grain was then extracted twice with 500-ml volumes of chloroform-methanol (70:30) in a Waring Blender. Solvent, after recovery by filtration, was evaporated and the residual solids were put into solution in 5 ml of olive oil. The fungal mycelial mats from the liquid media (one flask each) were treated similarly. The supernatant fluids from the YES and RT media were each extracted twice with 500 ml of CHCl3. The pH of the extracted YES supernatant fluid was then adjusted from 5.9 (final pH after incubation) to 2.0 with concentrated HCl and again the fluid was extracted twice with two 500-ml volumes of CHCl3; these solvent extracts were also combined, solvent was removed, and the residual solids were dissolved in 5 ml of olive oil. For acute toxicity studies, 20-g mice were injected (ip) with 0.2 ml of these olive oil solutions. Additional corn and sorghum which had been inoculated with *H. maydis* NRRL 3797 and incubated at 28 C was fed to mice as their only ration for 4 weeks; they then were fed normal ration for 2 months.

Production, isolation, and partial purification of *H. maydis* toxin. To produce large quantities of toxin, 32 Fernbach flasks, each containing 300 g of sorghum adjusted to 50% moisture, were inoculated with *H. maydis* NRRL 3797 and were incubated statically at 28 C for 2 weeks. The highly molded grain was pooled and extracted with 60 liters of CHCl3 plus 16 liters of CH3OH for 2 hr in a large stirred mixing tank. After settling, the reddish aqueous-methanol layer was siphoned off and discarded. It had been tested previously for toxicity by ip injection into mice, with negative results. The dark-green CHCl3 layer was concentrated to 1 liter in an all-glass pilot-plant evaporator and then was added slowly to 40 liters of cold pentane hexane. A dark-green precipitate, previously determined to be nontoxic by ip injection in mice, was filtered off and discarded. The pentane-hexane solution was concentrated to about 1 liter of a heavy dark-black oil from which insoluble substances were removed by centrifugation; this is referred to as residue A in the text. Uninoculated sorghum was treated similarly, and the pentane-hexane-soluble material is called residue B.

All residues were checked for sterility by plating 1 drop of each residue on Brain Heart Infusion agar and on potato-dextrose agar, and then incubating the plates at 37 C.

Column chromatography. One hundred-milliliter portions of the heavy oil (residue A) were placed on a Florisil (60 to 100 mesh) column (10 by 50 cm) and developed successively with 2-liter additions of hexane, CHCl3, and CHCl4 to which 10% increasing increments of acetone were added; fractions were collected in 200-ml quantities. The toxic fractions as determined by mouse assay, which were eluted with CHCl3 and CHCl3-acetone (90:10), were combined, concentrated, and chromatographed on a second Florisil column, to which was added 2 liters each of hexane, benzene, and benzene plus 10% incremental additions of ethylacetate.

Thin-layer chromatography. Thin-layer plates of 0.25-mm thickness and preparative plates of 0.75-mm-thick Silica Gel G-HR containing CaSO4 binder (Brinkmann Instruments, Inc., Westbury, N.Y.) were prepared on glass plates and activated at 110 C for 1 hr. Plate development was with benzene-ethyl acetate (70:30); detection was effected either by exposure to iodine fumes or by spraying with a modified Dittmer-Lester reagent (8).

Mouse studies. Mice used in this study were 11 to 12-g. CF-1, weanling, female mice (Carworth Farms, Portage, Mich.).

Five milliliters of olive oil was mixed with 2.5 ml of residues A and B, and 0.1 ml of this mixture was injected (ip) in each of 11 mice and subcutaneously in 1 mouse. Six mice, given 0.1 ml of olive oil only, served as controls.

Rat studies. The residue suspensions used for rats were the same as those used for the mice. The rats used were 40- to 50-g Sprague-Dawley white weanling females (Simonsen Laboratories, White Bear Lake, Minn.).
Six rats were each given 0.5 ml of the suspension (2.5 ml of residue A in 5 ml of olive oil) ip, and three rats were given 0.3 ml of the suspension ip. Three additional rats were given 0.5 ml of olive oil ip and served as controls. The liver, heart, and kidneys were removed from all rats at postmortem examination for histopathological examination.

Swine studies. All pigs tested were Yorkshire-Chester White cross, naturally farrowed, SPF, 2 months old, weighing approximately 16 kg; they were obtained from the National Animal Disease Laboratory herd. Pig A was male and pigs B, C, and D were female. Pig A was given 25 ml of residue A via a stomach tube. To flush the residue from the stomach tube, 15 ml of olive oil followed by an equal volume of air was passed through the stomach tube. Pig B was given 3 ml of residue B (equivalent amount of sorghum as used for obtaining 25 ml of residue A) as described for pig A. Pig C was given 5 ml of residue A ip, and pig D was given 3 ml of residue B by the same route.

Rabbit skin tests. Rabbit skin tests were conducted on New Zealand White rabbits weighing 2 kg. Skin sites on three rabbits were shaved, and 0.15 ml of residue A was applied over a 4-cm² area daily for 1 to 4 days. These sites were observed for dermonecrotic reactions, and specimens were taken for microscopic examination. One rabbit was treated in a similar manner with residue B.

Antibiotic tests. Antibiotic tests against bacteria, yeasts, and fungi were carried out by the method of Lindenfelser and Ciegler (6) and against tissue culture by the procedure of Perlman et al. (7).

RESULTS

Toxicity of extracts of H. maydis-blighted corn plants. Mice injected twice weekly for 4 weeks with solvent extracts of heavily blighted corn leaves exhibited no signs of acute toxicity and demonstrated the same weight gains as control mice. After 6 months, all mice were sacrificed; there was no evidence of chronic toxicity or abnormal gross pathology.

Preliminary toxicity studies on Helminthosporium isolates. Data in Table 1 show that all cultures assayed, except for H. carbonum race I NRRL 3798 and race II NRRL 3799, produced toxic material. Based on the rapidity and number of mouse deaths, sorghum grain appeared to afford a better substrate for toxin production than corn. Among the cultures tested, H. maydis NRRL 3797 was arbitrarily chosen for further investigation.

Toxicity of extracts of H. maydis (NRRL 3797) grown on natural and artificial media. On both the natural and artificial media, H. maydis produces a toxin which can be found within the mycelium and in the cell-free culture filtrate (Table 2). Adjusting the pH of the extracted supernatant fluid to pH 2 and a second extraction did not result in additional toxin extraction. Toxin production occurred at both 20 and 28°C, but in corn, the higher temperature appeared to be more favorable for toxin production. Extracts of control grains and media were nontoxic.

The moldy grain which was fed to mice was initially rejected, but after 1 to 2 days it was consumed readily. No signs of toxicity were noted in any of the mice during the 3-month experimental period.

Toxicity evaluation of H. maydis (NRRL 3797) cultured on sorghum grain: mouse studies. The 11 mice given the ip injection of residue A appeared depressed and were lethargic within 5 min after inoculation. Within 15 min they were incoordinated, their eyes were closed, and occasional tremors were noted. Dyspnea was observed in most of the mice 30 min after inoculation. These signs were observed in the mice until they died. The earliest deaths occurred at 4 hr after inoculation, and all mice inoculated ip died within 12 hr after inoculation. Liver and kidney tissues from four of these mice were fixed in 10% Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopic examination of these sections revealed a fibrinous peritonitis over the liver and a subcapsular zone of necrosis and neutrophilic infiltration two to three cells deep. No other significant alterations were observed in liver or kidney sections.

The mouse inoculated subcutaneously was killed 2 weeks after inoculation. At the postmortem examination there was a focal area of necrosis at the site of inoculation. Microscopically, a chronic, focal, suppurative, necrotic dermatitis which extended into the subcutaneous tissue was present. No signs of illness were observed in any of the control mice during the 1-week experimental period, and no lesions were found at postmortem examination.

Rat studies. Lethargy was noted in all rats injected with residue A within 5 min after inoculation. All rats given 0.5 ml of inoculum died after 32 hr, another died after 56 hr, and the remaining rat was killed 1 week after inoculation. At the postmortem examination an excess of clear, light-yellow fluid was found in the peritoneal cavity, and adhesions were found between the diaphragm and liver. Microscopic alterations in rats given 0.5 ml of inoculum consisted of a fibrinous peritonitis over the liver and a subcapsular zone of necrosis and neutrophilic infiltration. There was also a diffuse suppurative inflammation in the

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Table 1. Toxicity of Helminthosporium strains to mice

| Fungus                        | Medium grown upona                                                                 |
|-------------------------------|------------------------------------------------------------------------------------|
|                               | Corn (deaths/no. of mice)                                                                 |
|                               | Sorghum (deaths/no. of mice)                                                        |
|                               | 18 hr | 48 hr | 18 hr | 48 hr |
| H. carbonum race I NRRL 3798 | 0/2   | 0/2   | 0/2   | 0/2   |
| H. carbonum race II NRRL 3799| 0/2   | 0/2   | 0/2   | 0/2   |
| H. maydis race O NRRL 5127   | 1/2   | 2/2   | 2/2   | 2/2   |
| H. maydis race T NRRL 5128   | 2/2   | 2/2   | 2/2   | 2/2   |
| H. maydis race T NRRL 3797   | 2/2   | 2/2   | 2/2   | 2/2   |
| S. oryzae race Tb NRRL 5201  | 0/2   | 2/2   | 2/2   | 2/2   |
| H. maydis race Tb NRRL 5202  | 0/2   | 2/2   | 1/2   | 2/2   |
| H. maydis race Tb NRRL 5203  | 0/2   | 0/2   | 2/2   | 2/2   |
| H. maydis race Tb NRRL 5205  | 0/2   | 2/2   | 2/2   | 2/2   |
| H. maydis race Tb NRRL 5206  | 0/2   | 2/2   | 2/2   | 2/2   |
| H. maydis race Tb NRRL 5204  | 1/2   | 2/2   | 2/2   | 2/2   |

a Cultures were grown on the grains for 2 weeks at 28°C and solvent was extracted. Extracted solids were dissolved in 2.0 ml of olive oil, and 0.1 ml was injected ip. 

Induced substantial blighting on PAG hybrid 5 × 297. R. R. Nelson, Pennsylvania State University, kindly made race determinations for these strains.

Table 2. Effect of ip injections of crude extracts of Helminthosporium maydis NRRL 3797-inoculated laboratory media and grains

| Substrate              | Temp (C) | Deaths/no. of miceb injected (24 hr) |
|------------------------|----------|-------------------------------------|
| Corn                   | 28       | 9/10                                |
| Sorghum                | 20       | 2/10                                |
| YES mycelia            | 28       | 4/5                                 |
| YES supernatant fluid  | 28       | 4/5                                 |
| YES supernatant fluid, adjusted pH 2 | 28 | 0/5                                 |
| YES mycelia            | 20       | 5/5                                 |
| YES supernatant fluid  | 20       | 4/5                                 |
| YES supernatant fluid, adjusted pH 2 | 20 | 0/5                                 |
| RT mycelia             | 28       | 4/5                                 |
| RT supernatant fluid   | 28       | 5/5                                 |
| RT mycelia             | 28       | 0/5                                 |
| RT supernatant fluid   | 20       | 5/5                                 |
| Corn control           | 28       | 0/5                                 |
| Sorghum control        | 28       | 0/5                                 |
| YES medium control     | —        | 0/5                                 |
| RT medium control      | —        | 0/5                                 |
| Solvent control        | —        | 0/5                                 |

b Mice were injected with 0.1-ml amounts of propylene glycol solutions of the crude solvent extracts.

Swine studies. No signs of illness were observed in any of the control rats during the 1-week experimental period, and no lesions were found at the postmortem examination.

Immediately after inoculation, pig C was unable to stand. This pig appeared depressed and was lethargic 1 hr after inoculation. By 3 hr, the pig had vomited three times and was ataxic. Vomition occurred two more times within 6 hr after inoculation and at least twice more before the pig was found dead at 12 hr after inoculation. Except for some tremors, no signs of illness were detected in pig D during the first 1.5 hr of observation following inoculation. Pig D was killed 2 days after inoculation.

The essential result of ip injection of residues A and B into pigs C and D was the establishment of an exudative, fibrinous peritonitis. There was 400 to 500 ml of clear, light-brown fluid in the peritoneal cavity of pig C, numerous petechia on the parietal peritoneum, strands of black material over the spiral colon and around the liver and stomach, and scattered fibrin strands throughout the peritoneal cavity. The mesenteric vessels and those of the serosal surface of the entire intestinal tract were engorged. No other significant changes were seen in liver, kidney, or heart. In rats given 0.3 ml of inoculum there was suppurrative, fibrinous peritonitis over the liver with no significant changes in the remainder of the liver, omentum.
were markedly congested. The mucosa of the fundic area of the stomach was congested and contained petechia and ulcers. A 5 to 6-cm area of the mucosal surface of the duodenum was severely congested with numerous petechia. The mucosal surface of the entire small and large intestine was congested and contained occasional petechia. There was mild perirenal edema. All other organs examined had no visible lesions. Pig D had approximately 100 ml of light-yellow fluid in the peritoneal cavity. There were a few yellow adhesions between the spiral colon and parietal peritoneum, spleen, and omentum. Histopathologic examination of tissues from pig C revealed subcapsular necrosis of hepatocytes with infiltration of neutrophils into this layer of hepatocytes. There was a mild fibrinous peritonitis, edema, and mild supplicative inflammation of subperitoneal tissue, and congestion of the adrenal cortex. No significant changes were apparent in the gastrointestinal tract, adrenal gland, kidney, or liver of pig D; however, there was a pronounced fibrinous peritonitis undergoing organization.

Rabbit skin tests. Erythema of the skin developed after three daily applications of the residue. This progressed to a scaly-to-crustiy surface at the site of application. Histopathologic examinations of skin sections revealed that the epidermis was thickened (twice normal) and was hyperkeratotic 24 hr after a single application of residue A. Microabscesses developed between the keratinized layer and the underlying stratum granulosum. A mild congestion and neutrophilic exudation were present in the dermis. These changes became more pronounced following successive applications of extract so that, by day 4, a crust of keratinized epithelium and supplicative exudate covered the epithelium; mild congestion and suppuration were present in the dermis. Epithelial necrosis was not observed. No changes were observed in a rabbit treated similarly with residue B.

Antibiotic tests. Residue A showed slight antimicrobial activity against Staphylococcus aureus, Bacillus subtilis, Mycobacterium phlei, and Mucor ramannianus, but no activity against Brucella bronchiseptica, Saccharomyces pastorianus, Candida albicans, or the tissue culture line, Eagle's KB cells. Residue B, the control, exhibited no antimicrobial activity.

Toxin isolation. Despite repeated column chromatography under various conditions, separation of a toxin from residue A could not be achieved. The toxic fraction after thin-layer chromatography (TLC) showed the presence of four iodine-positive substances at Rf values of 0.19, 0.30, 0.40, and 0.44. Only the substance at Rf 0.30 was lethal when injected ip into mice. Various solvent-solvent fractionations were likewise unsuccessful. However, scraping the toxic band off of several preparative TLC plates and eluting the scrapings with CHCl3 and CH3OH resulted in isolation of a white amorphous substance that showed lipoidal physical properties. This substance was rechromatographed by preparative TLC to give a final yield of 10 mg. On TLC, only one spot was detected. However, after hydrolysis by the BF3-methanol method (Applied Science Laboratories, Bulletin no. 17) and gas-liquid chromatography (GLC) on 5% SE-30 columns [6 ft by ½ inch (approximately 1.8 m by 0.32 cm); Hewlett-Packard, Skokie, Ill.], the possible presence of three additional substances in very small quantities was revealed.

On TLC plates, the toxin band took up iodine vapors, did not react with ammonia fumes, and was negative to the FeCl3 and ninhydrin tests; the anisaldehyde test, indicative of a sugar moiety, was positive, as was the diphenylamine test indicative of a glycolipid (8); the modified Dittmer-Lester reagent (11) for phospholipids was also positive. Further evidence for the presence of a sugar moiety was obtained by hydrolysis of the toxin with 2.0 N HCl in a sealed tube for 18 hr at 105 C. The hydrolysate was treated with a trimethylsilyl derivative preparation (Tri-Sil reagent; Pierce Chemical Co., Rockford, Ill.) and then analyzed by GLC. Results were inconclusive but indicative of a carbohydrate such as galactose. Efforts to isolate the toxin in pure form are continuing.

TLC of toxic cultures. TLC extracts of corn, sorghum, and mycelia, from liquid cultures that had been inoculated with the strains noted in Table 1, all showed the presence of the same four iodine-positive compounds found from H. maydis NRRL 3797. The only exception was H. carbonum race I and race II, extracts of which did not have these substances. These latter organisms were the only nontoxic ones of those examined.

DISCUSSION

Our data support accumulating evidence that H. maydis-invaded corn is probably not toxic under field conditions (1, 5, 10). However, H. maydis races O and T produce a compound that is toxic on ip injection and topical administration to various animals, but appears
to have no toxicity on administration by mouth or as a diet contaminant.

Preliminary studies on a semipurified toxin fraction indicate that the compound is a glyco-
phospholipid. Its lack of toxicity, when ingested by mouth, may be due to degradation by the intestinal microflora.

Histopathologic examination indicates that the glyco-phospholipid, on injection, acts as an irritant. This is further supported by the action of the substance when applied to rabbit skin; considerable exudation rather than a necrosis results. It is thus desirable to conduct a histopathologic examination of the skin to determine whether a necrosis occurs on skin testing of mycotoxins.

In the isolation of most mycotoxins, the lipid fraction is discarded, and only the effect of the pure toxin on the host is determined. Whether these lipoidal fractions may interact with more obviously overt mycotoxins remains to be determined.

Although our data indicate that *H. maydis* does not produce a compound that is toxic by mouth, we have shown that a number of other fungi isolated from blighted corn, such as *Trichoderma* and *Alternaria* species, produce extremely toxic substances (*unpublished data*). Potential interaction between the substances and the glyco-phospholipid produced requires further investigation. In addition, it should be noted that a compound nontoxic under normal conditions can become toxic when predisposing conditions arise. This has been amply demonstrated for a variety of antibiotics and drugs.

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